

OLLE ZETTERVALL

Antibody Activity in Monoclonal Immunoglobulin G

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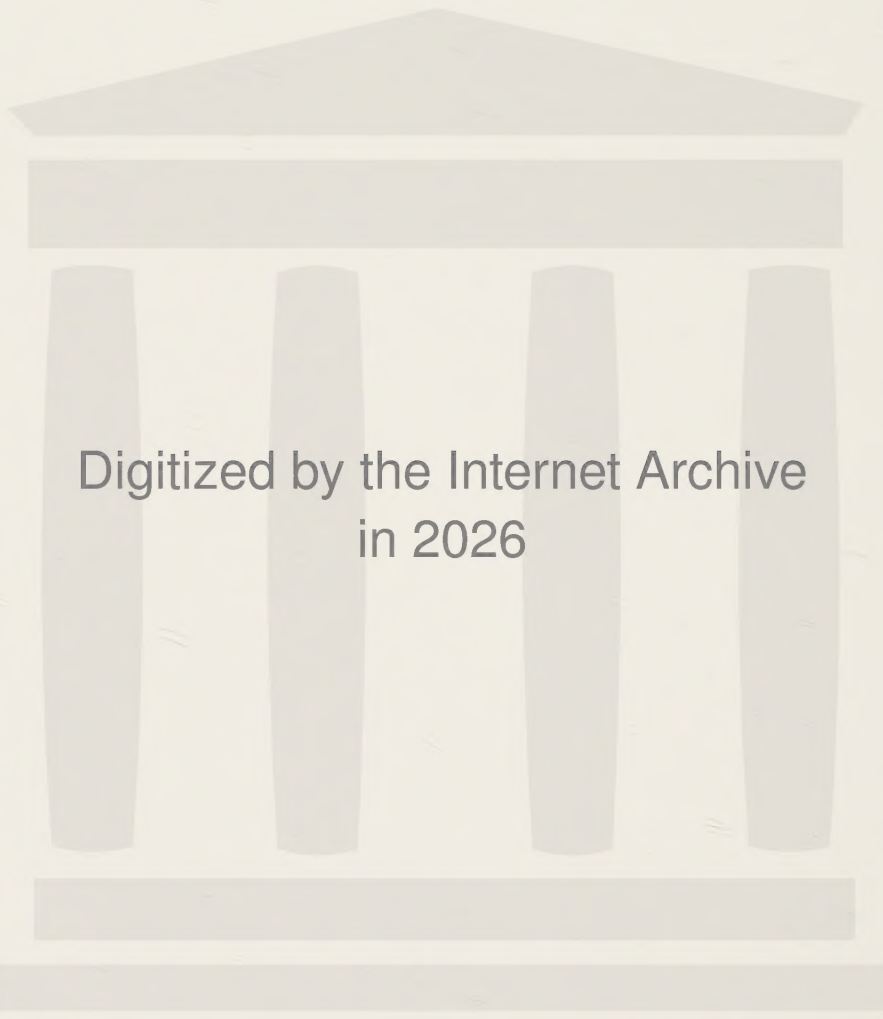
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FROM THE DEPARTMENT OF MEDICINE

(HEAD: PROFESSOR JAN WALDENSTRÖM, M.D.)

UNIVERSITY OF LUND, ALLMÄNNA SJUKHUSET, MALMÖ

AND THE DEPARTMENT OF NEUROLOGY

(HEAD: PROFESSOR RAGNAR MÜLLER, M.D.)

UNIVERSITY HOSPITAL, LUND, SWEDEN

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by

OLLE ZETTERVALL

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INTRODUCTION

Humoral antibodies belong to the collection of proteins known as the immunoglobulins. The immunoglobulins are defined by certain structural properties, some of which are shared by the majority of the members of this family of proteins. Our present concept of a basic four-chain structure originates from Edelman's (1959) work on chemically produced subunits and from Porter's (1959) work on enzyme cleavage products from immunoglobulin. The structure is thought to consist of 2 heavy and 2 light polypeptide chains united by covalent (disulfide) bonds and in addition by non-covalent forces. Isolated heavy and light chains may be prepared and subsequently recombined to four-chain molecules. When the chains are prepared from antibody-active immunoglobulin, the reconstituted four-chain molecules characteristically display antibody activity (Olins and Edelman 1964).

Heterogeneity among the immunoglobulins is immediately conceivable considering the wide spectrum of antibody specificities that may be elaborated in each individual. Immunoglobulin, *i.e.* antibody heterogeneity is, however, not limited to variations in antibody specificity. Thus human immunoglobulins display a large variety of electrophoretic mobilities. There are further differences in antigenic configurations on the heavy chains defining classes of which the quantitatively most predominant are IgG,* IgA and IgM. Recently 2 more classes have been described, *i.e.* IgD (Rowe and Fahey 1965 a, b) and IgE (IgNd) (Ishizaka, Ishizaka and Hornbrook 1966, Johansson and Bennich 1967). Heavy chain antigenic subclasses have been found in

IgG (Grey and Kunkel 1964, Terry and Fahey 1964), in IgA (Feinstein and Franklin 1966, Kunkel and Prendergast 1966, Terry and Roberts 1966, Vaerman and Heremans 1966), and in IgM (Harboe et al. 1965). Similarly antigenic differences have been recognised in the light chains and 2 main types are known, *i.e.* K and L. (Fahey 1963, Mannik and Kunkel 1963.) Genetically determined factors, Gm characters, are known in human IgG (Grubb 1956) and are associated with the heavy chains. Other allotypic factors (Inv) (Ropartz et al. 1961) are found on human light chains of type K. Electrophoretic heterogeneity, classes, subclasses and allotypic variants of IgG are also found in species other than man. The molecular weights of the immunoglobulins vary between approx. 900 000 for IgM down to 150 000 for IgG and IgA. Higher molecular weights of IgA due to polymerisation are also encountered. For a recent review, see Cohen and Milstein (1967). A further dimension of heterogeneity is apparent from studies on purified antibodies as a variation in the structure of the antibody combining site even in cases where a well-defined antigenic configuration has been employed. This has for instance been demonstrated as a heterogeneity of binding affinities for the antigen in certain anti-hapten antibodies (Eisen and Siskind 1964).

The use of homogenous, or almost homogenous immunoglobulins, *i.e.* M-components, has greatly facilitated the study of immunoglobulin structure. M-components are not normally found but occur, often in huge quantities, in a multitude of clinical conditions, malignant or not. The fact that it has been possible to correlate almost all findings obtained with M-components with properties of

* The terminology recommended by the World Health Organization (Bull. Wld Hlth Org. 30: 447, 1964; 33: 721, 1965; 35: 953, 1966) was followed.

normal immunoglobulins has created the belief that M-components are to be considered as representatives of normally occurring proteins, or even that the normal immunoglobulins could be regarded as a collection of M-components (Waldenström 1961, Kunkel 1965). In view of this antibody active M-components as models of ordinary antibodies have been looked for.

Rheumatoid factor activity may be demonstrated in certain IgM M-components (Kritzman et al. 1961) and cold agglutinin activity in others (Christensen et al. 1957, Fudenberg and Kunkel 1957). Anti-lecithin activity in an IgM M-component was reported by Killander, Killander, Philipson and Willén (1967) and anti-alpha and beta-lipoprotein activity in an M-component of IgA class by Beaumont (1967). A serum with an IgA M-component and extremely elevated anti- α -staphylolysin activity has been described by Mansa and Kjems (1965). Dammacco and Clausen (1966) have found an M-component giving precipitin reaction with staphylococcal and pneumococcal extracts.

The presence of antibody activity in IgG M-components was inferred by Waldenström (1961) from the finding of a serum with such M-component and excessive antistreptolysin-O titre. Besides this case (patient EN) another serum (AO) with the same characteristics was found by Waldenström et al. (1964) in a survey of 188 sera containing M-components. This collection also contained 2 sera with considerably increased anti-thyroglobulin activities. Other examples of a high antistreptolysin activity associated with an IgG M-component have been given by Badin and Cabau (1964), Kronvall (1967) and by Egelund Schmidt et al. (1968) and more cases may be among the further 6 instances of M-component together with elevated anti-streptolysin-O activity briefly given in the last mentioned paper.

Anti- α -staphylolysin activity in an IgG M-component was described by Mansa and Kjems (1965). A similar serum (IN) was discovered by Winblad.* Recently Eisen et al. (1967) reported a G-myeloma globulin reacting with a hapten, *i.e.* ϵ -2, 4-dinitrophenyl-L-lysine.

No uniform clinical picture seems to be associated with the presence of a high antibody activity and an IgG M-component. The absence of a definite history of exposure to the appropriate antigen has been explicitly stressed in some cases (Waldenström et al. 1964). A clinical survey of the cases AO, EN and IN will be given by Hällén et al. (1968).

The finding of reactivity of an M-component in an antibody assay system does not necessarily prove that the globulin is to be regarded as a true antibody. Since in practice a thorough history of immunisation is impossible to obtain for an individual the question whether the M-component is a true antibody or not could only be solved by study of the intrinsic properties of the protein. In the final analysis this may require an elucidation of the M-component's primary structure, which to date has not been obtained for any multichain immunoglobulin. However, if an antibody active M-component is found not to conform with accepted functional and structural criteria for ordinary antibodies, its activity will probably represent a pathological phenomenon. Conversely, each finding of a typical antibody property in the protein increases the probability that it represents an ordinary antibody globulin.

In this work the IgG M-components AO, EN and IN are investigated for the following properties: a) antibody activity in the proteins, b) localisation of antibody activity in their papain fragments, c) formation of antibody active molecules at recombination of their polypeptide chains, and d) binding of their presumed antigens.

* The Sample of serum IN used in the present investigation was the generous gift of professor S. Winblad, Malmö.

I. Materials and methods

In this Chapter are described some methods especially developed for this work, *i.e.* an acrylamide gel electrophoresis system suitable for analysis of heavy and light polypeptide chains, the use of a low molecular marker for identification of molecular size in gel filtration experiments, and diffusion-in-gel techniques for determination of antistreptolysin-O and anti- α -staphylolysin activity.

In addition are given standard materials and methods commonly used in the investigations of the Chapters II—V.

Additional methods or minor departures from the procedures given in this Chapter are stated in each instance.

MATERIALS

Patient sera AO, EN and IN are described in Chapter II. The serum SE had been obtained from a patient with a history of throat infection preceding arthralgia. It had an AST titre of 4500 U/ml.

Serological reagents. Streptolysin-O, α -staphylolysin broth and horse standard anti- α -staphylolysin serum was obtained from the National Bacteriological Laboratory, Stockholm. Standard human anti-streptolysin-O serum was from the Danish State Serum Institute, Copenhagen.

Protein preparations. Pooled normal human IgG, prepared by ion exchange chromatography of a Cohn fraction, was obtained as a lyophilisate from Kabi AB, Stockholm, Sweden. On immunoelectrophoresis at a protein concentration of 1 g/100 ml employing polyvalent anti-human serum, only IgG could be demonstrated. This preparation is designated IgG Kabi, or pooled normal IgG. Human immunoglobulin against staphylococci was obtained from the National Bacteriolog-

ical Laboratory. The preparation was purified by ion exchange chromatography (DEAE SEPHADEX A-50 (Pharmacia), 0.0175 M phosphate, pH 6.50). It consisted of pure IgG (IgG ASTA) by the same criteria as valid for IgG Kabi. IgG AL and IgG EG had been prepared by ion exchange chromatography from 2 human myeloma sera and was a gift from Dr. J. Sjöquist, Uppsala. IgG AL is of type GK and IgG EG of type GL.

Human transferrin was obtained from Kabi AB and bovine serum albumin (BSA) from Armour Pharmaceutical Co. Ltd., Eastbourne, England.

Isotopes. ^{125}I was obtained from the Radiochemical Centre, Amersham, England. ^{131}I was obtained from the same source, or from AB Atomenergi, Studsvik, Sweden. The preparations were free from reducing agents. Radioiodinated human serum albumin (RIHSA) was from the last mentioned manufacturer.

Chemicals. With the exception of sodium dodecyl sulphate (SDS) which was of Swedish Pharmacopoeia quality, all chemicals were of reagent grade, or better; they were used as delivered by the manufacturer.

METHODS

Analytical and preparative methods

Spectrophotometry was carried out in a Beckman DU or Beckman B spectrophotometer, 1 cm quartz cuvettes were used at ultraviolet and sometimes at visible wave-lengths; otherwise 1 cm glass cuvettes were employed.

Determination of protein concentration. For solutions of purified immunoglobulins and of polypeptide chains of immunoglobulins the extinctions at 280 nm were used. One and the

same extinction coefficient was assumed for these kinds of material, *i.e.* $E_{1\text{ cm}}^1 \%$ = 13.6, the figure given for human gamma globulin by Crumpton and Wilkinson (1963). For other types of protein solutions, and sometimes for those already described, a modification (Eggstein and Kreutz 1955, Legget Bailey 1962) of Lowry's et al (1951) method was employed. Samples of IgG Kabi solutions of known protein concentrations were used as standards. Determination of total serum protein is described in Chapter II.

Agar gel electrophoresis was performed by Wiemes (1959) technique.

Paper electrophoresis was carried out according to Laurell, Laurell & Skoog (1956) with modifications regarding staining (Zettervall et al. 1966).

Horizontal starch gel electrophoresis. Poulik's (1957) tris citrate buffer system was used. The gels were prepared in $0.5 \times 11 \times 26$ cm Perspex moulds; samples (20 μ l) soaked into 0.5×1 cm strips of Whatman No. 3 filter paper, were inserted into the gel and electrophoresed at $+4^\circ\text{C}$ for 16 hr at a field strength of about 4 V/cm. Amido Black B (0.1 per cent w/v) in methanol-acetic acid-water (5 : 1 : 5 v/v) was used for staining (10 min.) of the sliced gel. Solvent without dye was used to remove any excess stain.

Acrylamide gel electrophoresis. Maizel's (1966) method was modified in that urea was included in sample and gel buffers; further, gel plates were used instead of columns. The changes were introduced to facilitate dissolution of lyophilised heavy chains, difficult to dissolve in detergent alone, and to enable comparison of samples, analysed in one and the same gel. The actual procedure was as follows. To make up 1000 ml of gel solution, acrylamide 50 g SDS 1 g, N,N-bismethylene acrylamide 1.3 g and urea 480 g were dissolved in enough 0.1 M, pH 7.2 phosphate buffer to give the assigned volume. This solution was still usable after several months storage in the cold.

Polymerisation was achieved by addition, to final concentrations, of 0.05 per cent (w/v) N,N,N',N'-tetramethylethylene diamine and 0.075 per cent ammonium persulphate. After rapid mixture of the constituents, 125 ml of the solution was poured into a $0.3 \times 11 \times 26$ cm Perspex mould. A glass plate was immediately placed over as a cover, care being taken to avoid air between the glass and the gel solution. After 1–24 hrs. the cover was removed and the gel was ready for use. The gel contains urea at approximately 8 M and SDS at about 0.1 per cent (w/v). The protein samples, lyophilised from 0.1 M formic acid, were dissolved in sample buffer, made up by dissolving, in 0.1 M, pH 7.2 phosphate, urea and SDS to final concentrations of 8 M and 1.2 per cent (w/v), respectively. The samples were kept at room temperature for 1–3 hr before electrophoresis. This time was arbitrary. The samples (10 μ l) were applied on 2.5×10 mm Whatman No. 3 filter paper strips then applied to slits in the gel. Double layers of Whatman No. 3 filter paper were used as bridges to the buffer troughs, filled with 0.1 M phosphate, pH 7.2 with 0.1 per cent (w/v) SDS. The horizontal gel was covered with Saran film. Electrophoresis was carried out at room temperature at a field strength of initially about 2 V/cm, rising in 12 hrs. to about 3 V/cm. To visualise the protein pattern, the gel was stained without slicing, using the same procedure as for starch gels. After further destaining for a few hrs. in 1 per cent (v/v) acetic acid gels have been dried under stretched water permeable cellophane. So treated, they are suitable for filing. When radioactive proteins, such as radioiodine labelled heavy and light chains, are to be analysed, the gel is easily cut into fractions for counting.

Preparative electrophoresis in Sephadex G-25 Fine. Buffer of the composition 0.05 M diethylbarbiturate, 0.01 M diethylbarbituric acid and 0.05 M sodium acetate, pH 8.6 was used in gel and electrode vessels. The gel was prepared on a glass plate in a block of dimensions $0.3 \times 10 \times 25$ cm. The sample, made up with dry Sephadex to a thick slurry, was applied

to a 0.5×6 cm transverse slit in the gel block. Double Whatman No. 3 filter papers were used as bridges to the electrode vessels. Electrophoresis was carried out at $+4^\circ\text{C}$ in a moist chamber at a field strength of about 5 V/cm. Running times are given in each separate case. After electrophoresis gel fractions of approximate dimensions 0.5×6 cm were scraped from the plate, and protein was eluted in standard volumes of buffer. Protein in the eluates was assayed with the Lowry method.

Cellulose acetate electrophoresis, analytical or preparative, was performed as described elsewhere (Zettervall et al 1966).

Ion exchange chromatography. Either DEAE Sephadex A-50, CM-Sephadex C-50 (Pharmacia AB, Uppsala, Sweden), or DEAE-Cellulose (Serva Entwicklungslab., Heidelberg, W. Germany) were used as ion exchangers in column procedures. Column dimensions, buffers and flow rates are given in each instance. In cases where elution was carried out with a gradient procedure, cylindrical open beakers of equal dimensions were used as mixing chamber and limit buffer reservoirs, respectively. The beakers were connected with a siphon and buffer was pumped from the mixing chamber to the column.

Gel filtration employing Sephadex G-200, particle size 40–120 μ (Pharmacia) was performed in either of the following buffers: tris-HCl, 0.1 M, pH 8.0; phosphate buffered saline (PBS): 0.075 M phosphate-0.075 M sodium chloride, pH 6.5; or 0.1 M formic acid. The column length to diameter ratio was 20 : 1, or higher. Buffer flow, maintained with a peristaltic pump, was 3 cm/h, or lower. Columns equilibrated with tris-HCl or PBS were operated in the cold, whereas those with formic acid were used at room temperature. Fractions were read at 280 nm and/or assayed for radioactivity.

In the gel filtration experiments with Sephadex G-200-PBS riboflavin was usually included in the samples as an internal standard. A knife's point of the substance (M.W. 356.78) was mixed with the sample which was

then cleared from undissolved dye by centrifugation. After completion of chromatography, the visibly yellow fractions were read at 280 nm to define the peak riboflavin fraction, or this could be found by direct inspection. Elution volumes for protein and riboflavin peaks were then measured or calculated from flow rates and fraction numbers. The index, R_{ribo} could then be calculated for each material eluted, by inserting the respective elution volumes for the peak fractions into the definition formula, $R_{\text{ribo}} = \text{elution volume}$

of peak/elution volume of riboflavin peak. When dilute (1 : 1, saline) human serum was analysed on three columns (diameter 0.9–1.1 cm, length 90 cm) the figures for R_{ribo} were: albumin 0.637–0.666; IgG 0.498–0.545, and void peak 0.329–0.356. These ranges, calculated as percentages of the respective means are 4.5, 8.9 and 7.8, respectively. Approximately equally narrow ranges were obtained with radioiodinated IgG, isolated heavy or light chains or with papain split products from IgG. The figures for the respective R_{ribo} will be given in Chapters III and IV. R_{ribo} seems to be a useful character-

istic of molecular size at least in connexion with the buffer and Sephadex grade employed. The index facilitates comparison of results from different gel filtration experiments. Gel filtration in Sephadex G-25 is described in Chapter IV.

Counting of radioactivity was performed in a Packard Auto-Gamma well crystal spectrometer. With the settings employed counting efficiency was about 28 per cent for ^{125}I and 18 per cent for ^{131}I . In giving the results corrections have been made for decay, natural background and, when the 2 isotopes were simultaneously counted, for interference with the ^{125}I channel from the ^{131}I channel. No significant background from ^{125}I on the ^{131}I counts was apparent. Standard preparations were included to enable comparison of results obtained at different countings.

Immunochemical procedures.

Preparation of antisera:

Immunisation of rabbits. 2 volumes of antigen solution, containing 2–3 mg of protein, was emulsified with 1 volume of Freund's complete adjuvant. A rabbit was injected subcutaneously with the mixture which was dispersed over 3–4 injection sites. Rabbits were injected once every 1–2 weeks and serum was harvested after 5 months or longer.

Absorption technique. To 1 volume of antiserum was added cross reacting antigen either in <1/10 vol. as solution or as lyophilisate. The mixture was incubated at 37°C for 1 hr and subsequently overnight in the cold. Any precipitate was removed by centrifugation.

Antisera. The following were prepared as described above (abbreviations in brackets).

Anti-IgG: 2 sera were used; each proved specific as anti-IgG when used in immunoelectrophoresis of normal human serum. One of the sera was absorbed with normal human light chains. When tried at different dilutions in immunodiffusion against serial dilutions of normal human serum in conjunction with specific anti-IgA and anti-IgM sera the absorbed serum (*anti-IgG A*) proved specific against IgG; the other serum (*anti-IgG B*), was used without absorption. *Anti-normal human γ -chain, (anti- γ -chain),* the serum was absorbed with normal human light chains. No cross reaction was obtained with light chains in immunodiffusion.

Anti-normal human light chain, (anti-light chain), the serum was absorbed with normal human heavy chains. No reaction with heavy chains was discernible in immunodiffusion.

Anti-individual IgG, against IgG from the patient sera AO, EN and IN, (anti-IgGAO, EN and IN, respectively). These antisera are described in Chapter II. *Anti-bovine serum albumin (anti-BSA).* The serum gave one line against BSA at immunodiffusion.

Specific rabbit *anti-transferrin, anti-IgA, anti-IgM* sera and polyvalent *anti-human rabbit serum (anti-human)* were obtained from Behringwerke, Marburg-Lahn, W. Germany. Specific

rabbit *anti-light chain type K (anti-K) and L (anti-L)* sera were a gift from Dr. I. Berggård, Uppsala.

Immunodiffusion and immunoelectrophoresis was carried out according to the modified Ouchterlony (1948) and Scheidegger (1955) methods as described by Link (1967).

Single radial immunodiffusion. A modification (Fahey & McKelvey 1965) of the original method of Mancini et al (1963) was used with the exception that wells of 3 mm diameter were employed.

Tube method for determination of anti-streptolysin-O (AST) activity. Titration was performed according to Liao's (1951) method with certain modifications. As in the original technique, doubling dilutions of the sample to be titrated was reacted with a standard amount of reduced streptolysin, diluted to 1 combining unit/ml. After addition of washed sheep red cells and incubation, haemolysis was estimated by reading the centrifuged and suitably diluted samples at 525 nm. The percentage of haemolysis was then calculated from a standard curve relating optical density and haemolysis percentage. The dilution of the sample to give 50 per cent haemolysis was interpolated with the nomogram of Liao, relating probit of haemolysis percentage to dilution of the sample. This interpolated dilution constituted the end point of the titration.

The actual procedure at titration was as follows. Five conical glass centrifuge tubes, numbered 1 to 5, received 0.5, 0.75, 0.875, 0.938 and 0.970 ml of phosphate buffered saline (PBS), respectively from a 1 ml pipette. Of the solution to be tested 0.5, 0.25, 0.125, 0.06 and 0.030 ml was added to the respective tubes to make up 1 ml of doubling dilutions. A slight systematic departure from the appropriate dilution factor (*i.e.* 2) is evident for the step from tube No. 3 to No. 4. A 1 ml pipette was used for the additions to tubes No. 1–3 and a 30 μ l constriction pipette for No. 4 and No. 5. Meanwhile, streptolysin broth was reduced for 30 min. at room temperature by addition of 1/25 volume of

10 per cent (w/v) sodium pyrosulphite in PBS. After dilution with PBS to a concentration of 1 combining U/ml, 0.5 ml of the streptolysin was added to each of the serial dilution tubes, which were then rapidly shaken. The tubes were incubated for 30 min. in a 37°C waterbath. To each tube was then added 0.5 ml of 5 per cent (v/v) sheep red cells in PBS. After rapid mixing, haemolysis was allowed to continue for 60 min. at 37°C. After the addition of the red cells the tubes were again gently shaken after 15 min. Incubation was terminated by addition to each tube of 2 ml PBS. After mixing, the tubes were centrifuged and the clear supernatants were read at 525 nm.

The standard haemolysis percentage vs. OD₅₂₅ curve had been made up from known dilutions of a saponin haemolysate of 5 per cent (v/v) sheep red cells. A strict proportionality was apparent between degree of haemolysis and optical density. OD₅₂₅ corresponding to 50 per cent haemolysis in the actual AST titration system was 0.345. The sheep red cell suspensions to be used in the titrations were always adjusted to give an OD₅₂₅ of 0.345 ± 10 per cent at 50 per cent haemolysis.

The appropriate dilution of streptolysin broth to give a toxin concentration of 1 combining U/ml was obtained by allowing serial dilutions of reduced broth (1 ml) react (37°C, 30 min.) with a standard AST preparation (0.5 ml) containing 1 AST U/ml. Standardised sheep red cell suspension (0.5 ml) was then added. Incubation for haemolysis and its evaluation was carried out as for AST titrations. The streptolysin dilution giving 50 per cent haemolysis was interpolated from v. Krogh's (1916) equation. At this dilution the streptolysin broth was considered by definition to contain 0.5 streptolysin combining U/ml, *i.e.* 1 ml of the dilution was considered to neutralise the 0.5 AST units from the AST standard employed.

The influence on the result of the AST titration of the following factors were specially investigated: (i) the titre level (ii) variation of initial dilution, (iii) variation due to different

batches of sheep blood and streptolysin.

For study of factor (i) the following experiment was carried out. Dilutions of IgG Kabi in PBS, the protein concentrations of which differed by a factor of 2^n , $n=1, 2, 3$, were carefully made up. Each dilution was titrated 5 or 6 times. Assuming a perfect serial dilution technique at titration, identical titre figures will be obtained for the different IgG Kabi solutions when the results are corrected for the variation in initial dilution. Conversely, variability of the normalised titre values could be considered to reflect imperfections in the serial dilution method. In the statistical treatment of the results, standard deviations are estimated from the ranges (Tippet 1925); standard errors are calculated as $\sigma/\sqrt{n}$. The results were (i): The means of the different multiple titre determinations (corrected for initial dilutions) ranged between 6.06 and 6.99 AST U/ml; the standard deviation varied between 0.08 and 0.45, and the coefficients of variation between 1.3 and 6.5 per cent. The mean titre for the whole material ($n=23$) was 6.46, standard deviation 0.36 AST U/ml, coefficient of variation 5.6 per cent and the standard error was $0.36/\sqrt{23}=0.073$ AST U/ml. The mean titre for the determinations with end points in the interval between tubes 3 and 4 differed by 0.53 AST U/ml from the total mean of the material, a difference compatible with random variation ($p>0.05$). The differences obtained with the titre means from the other determinations were even lower. The type (ii) error was studied by titration of 4 different dilutions of IgG Kabi, making the ratios between the protein concentrations deviate from 2^n . The coefficient of variation was 10.1 per cent. The variation of type (iii) was obtained from titrations of standard preparations, always included at titration. Determinations taken at random from the protocols, of 6 standard IgG Kabi samples frozen in aliquots and used only once after thawing were employed; 3 different batches of lysin and several different batches of blood had been used in this series of determinations. The coefficient of variation was 7.2 per cent.

Using the highest coefficient of variation found for the type (i) error and then considering the 3 types of errors as independent the total coefficient of variation for the titration method was calculated as

$C = \sqrt{6.5^2 + 10.1^2 + 7.2^2} = 14.0$ per cent. This means that in the case 2 titre values differ by 50 per cent, or more of the higher value, the difference is probably real ($p < 0.02$).

Micro modification of the AST tube titration method. When small amounts of material were to be titrated, the reaction system volume was reduced tenfold, keeping the volumetric relation between the reactants the same as for ordinary titrations. The serial dilutions were made up separately with constriction pipettes and transferred to plastic Ellerman tubes. Conditions for the reaction between lysin and AST as well as for haemolysis were the same as those given for the ordinary method. The tubes were tightly capped during incubations. After haemolysis, 1 ml of PBS was mixed with the contents of the tubes, and the degree of haemolysis evaluated after centrifugation. The precision of the method was evaluated as for the ordinary method. The type (i) error was, as coefficient of variation, 4.9 per cent or less. For error of type (ii), the coefficient of variation was 13.8 per cent. Type (iii) error: 6 titrations of standard IgG Kabi preparations, whereby 2 batches of lysin and several of blood had been used, gave the coefficient of variation of 18.2 per cent. The total coefficient of variation, calculated according to the principles used for the ordinary titration method, was

$\sqrt{4.9^2 + 13.8^2 + 18.2^2} = 23$ per cent.

In polyclonal material, the correlation between the results of the ordinary and micro method appeared to be linear. The graphically determined regression line had the equation: $\text{titre}_{\text{ordinary method}} = 1.135 \times \text{titre}_{\text{micro method}} - 0.03$; However this may not be valid in monoclonal material (Chapter IV).

Diffusion-in-gel method for determination of AST activity. For titration of AST activity in micro-

litre quantities of material, scanning large numbers of samples as chromatographic fractions or for study of inhibition of AST activity by antisera, a plate method was developed. In principle, AST was allowed to diffuse in sheep blood-agar containing unreduced streptolysin. After reduction AST activity was visualised as zones of haemolysis inhibition; 5 ml of 3 per cent agar (Agar-Noble Difco) in PBS, at 56°C was mixed with 2 ml of 5 per cent sheep red cells, 5 U of streptolysin and PBS to 10 ml. The gel solution was rapidly poured on a strictly horizontal dry glass plate and the gel was allowed to set. The gel was ready for use in 15 min., or it could equally well be kept in refrigerator at least 5 days without damage. Holes were cut in the agar with the same technique as described for radial immunodiffusion plates. Samples, usually 5 μ l, were applied to the holes with the aid of Carlsberg or Marburg micropipettes. Diffusion was allowed to proceed for usually 5–10 hrs. in a moist chamber. Streptolysin in the plate was then activated by placing double layers of filter paper (Munktell No. 50) soaked in 10 per cent (w/v) sodium pyrosulphite in PBS carefully on the gel surface. After 7–10 min. the filter paper was removed. Haemolysis was continued until lysed areas appeared clear, or nearly so, and zones of haemolysis inhibition, if present, sharply demarcated. The plate was fixed by immersion in 10 per cent neutralised formalin—0.9 per cent (w/v) sodium chloride. After 12 hrs, or longer the plate was washed in distilled water for at least 1 hr. It was then dried with the same technique as used for immuno-diffusion agar plates. Two perpendicular diameters of the haemolysis inhibition zones were then read to the nearest 0.1 mm with a slide caliper and their means calculated. When AST titres were to be determined known serial dilutions of a solution of the material to be tested were applied onto the same plate as standards. AST solutions from one source could not necessarily be titrated with the aid of standard preparations of different origin since the type of relation between tube titres and zone dia-

when calculated as percentages of the corresponding true values. All titre values were recalculated as differences from the true values and expressed as percentages of these, and the material was statistically treated by the analysis of variance method. No obvious difference was found between the 2 titre level groups at the 5 per cent significance level. The mean was 4.35, standard deviation 11.7 and the standard error 2.14 per cent of the true value ($n = 30$). Thus, if judged from this experiment, a titre value obtained at a single determination does not probably deviate from the true value by more than $4.35 + 2.4 \times 11.7 = 33$ per cent ($p < 0.02$).

If the individual calibration curves are used, the coefficients of variation and the standard errors are approximately the same as obtained above. However, the variation of the differences of the means and the true values may be greater, up to 21 per cent of the true values.

Tube method for determination of anti- α -staphylo-lysin (ASTA) activity. Titration was performed with α -staphylo-lysin broth diluted to 0.1 combining U/ml. The same dilution buffer (PBS) and volumes of buffer, red cell suspension, toxin dilution and antibody solution as for the AST titration system was used. Conditions for reaction between toxin and antibody (37°C, 30 min.) were also the same for the two titration methods. However, a 2 per cent rabbit red cell suspension in PBS was used and haemolysis was allowed to develop for 60 min. at 37°C and subsequently for 3 hrs. in the cold. After centrifugation, haemolysis was evaluated without previous dilution of the tube contents. At 50 per cent haemolysis, OD₅₂₅ from the standard red cell suspension amounted to 0.495, and the red cell suspensions used in the titrations were made up to give this figure within 10 per cent at the same degree of haemolysis, due allowance for dilutions being taken.

For standardisation of staphylo-lysin, closely spaced dilutions (1 ml) of such was allowed to react with standard horse ASTA serum (0.5 ml) diluted to 0.1 international U/ml. Reaction time was 0.5 hr at temperature 37°C.

After addition of 0.5 ml of standard rabbit red cell suspension, haemolysis and assay of haemolysis were carried out as described above. The toxin dilution, resulting in 50 per cent haemolysis was interpolated with v. Krogh's equation. This degree of haemolysis was used to define the end point as in the actual titrations of ASTA activity.

The precision of the ASTA titration method was determined according to the same principles as used for the AST tube titration method. The results were: type (i) error, standard horse ASTA serum used; means of the multiple values for each dilution ranged between 31.2 and 32.1 ASTA U/ml (correct figure by definition 32.0 U/ml; the serum had been used for standardisation of the toxin employed). The standard deviation varied between 0.52 and 2.2 U/ml and the limits for the coefficient of variation were 1.6 and 7.0 per cent. The mean titre of the whole material was 31.7, the standard deviation 1.41 and the standard error 0.29 ASTA U/ml ($n = 24$); the coefficient of variation was 4.5 per cent. The means from the various tube interstices did not significantly differ from the total mean. Type (ii) error: titrations of 4 differing dilutions of standard horse ASTA serum, coefficient of variation 11.1 per cent. Type (iii) error: 5 determinations on IgG ASTA at different occasions using 2 batches of lysin and several different batches of blood, coefficient of variation 15.5 per cent. The total coefficient of variation, calculated as was the case for the AST tube titration method thus amounted to $C = \sqrt{7.0^2 + 11.1^2 + 15.5^2} = 20.3$ per cent. Since the upper limit for the coefficient of variation of type (i) has been used the actual precision is probably better. Considering the figure obtained it is inferred, that a difference between 2 titre figures, obtained at different occasions, and amounting to 70 per cent or more of the higher value is probably real ($p < 0.02$).

Diffusion-in-gel method for determination of ASTA activity. The method was used for similar purposes as was the AST plate method.

ASTA active material was allowed to diffuse in rabbit blood-agar plates after which staphylolysin was diffused into the system and neutralised around the points of application of samples. The plates were made up by pouring 10 ml of gel solution at 56°C into plane glass Petri dishes of diameter 9 cm. The gel, made up with PBS, contained (final concentrations) agar (Agar-Noble Difco) 1.5 per cent and washed rabbit red cells 1 per cent. The gel was allowed to set for 15 min. in a moist chamber. Plates were then ready for use, or they could be stored in a moist chamber at +4°C for at least 5 days before utilisation. Samples, usually of 10 μ l volume, were applied in wells of diameter 3 mm, cut with the technique described for AST plates. The plate was transferred to a humid atmosphere and diffusion was allowed to continue at room temperature for 5–10 hr. Staphylolysin (15 ml) diluted with PBS to 0.1 combining U/ml, was then poured on the gel layer. The plate was observed intermittently for about 1.5 hr; 4–5 times during that period the plate was gently rotated for a few seconds to induce mixing of the lysin, otherwise it was left strictly horizontal in a moist chamber at room temperature. When zones of haemolysis inhibition, if any, were readily discernible, the lysin solution was poured out from the dish and substituted with 10 per cent formalin–0.9 per cent saline. After 12 hrs. or more formalin treatment, the plate was washed for at least 1 hr. in distilled water. The agar layer was loosened from the Petri dish, transferred to a glass plate, and dried. Zone diameters were then measured as described for the sheep red-cell-streptolysin plates. Reading was best performed at indirect illumination with the agar surface pressed against a white background. A certain error is introduced at reading zone diameters since the outline of the zones is somewhat diffuse.

The precision of the method was investi-

gated along the same principles used for the AST plate method, *i.e.* with 2 plates, each with 2 equal sets of known serial dilutions for calibration and 2 different known test solutions randomly spread over the wells. Calibration was done with doubling dilutions of IgG ASTA, the ASTA titres of which differed between 5.89 and 0.37 U/ml. For each inhibition zone perpendicular diameters were measured and their means calculated. The 2 test solutions had the titres of 3.93 and 0.98 U/ml; 10 μ l quantities were pipetted of all solutions. The standard curves (zone diameters against log tube titre) appeared sigmoid. For studying the precision of the method the mean standard curves of the 2 plates were used. They were obtained by plotting against log tube titres the means of the respective diameters from the 2 calibration curves on the same plate. The statistical treatment of the data was the same as that used for the AST plate method. For both plates together the coefficients of variation of the 4 sets of values were 10–45 per cent, and the differences between means and true values in percentage of the latter 3.7–25 per cent. For the whole material the mean of differences between found values and true values was 20.2, standard deviation 36 and standard error 7.2 per cent of the true value ($n = 28$).

Miscellaneous

Storage of proteins was usually at –15 to –20°C. However, some batches of the standard anti-streptolysin-O and anti- α -staphylolysin sera were kept at +5°C, others were frozen in aliquots and used only once after thawing. Heavy and light chains in 0.1 M formic acid were stored at +5°C.

Concentration of protein solutions was either performed by ultrafiltration in collodion bags (Membranfiltergesellschaft, Göttingen, Germany) at +4°C, or by lyophilisation.

II. Serological, immuno- and physico-chemical characterisation of the sera AO, EN and IN

Some features of the sera AO and EN have been described in earlier publications (Waldenström 1961, Waldenström et al 1964, Zettervall et al 1966). It was shown that the excessive AST activity of these sera was concentrated to the electrophoretic gamma-globulin region. Further it was found, that inside this region the antibody property appeared to be condensed in the area of the M-component. In fact, a nearly quantitative recovery of serological activity was found at preparative cellulose acetate electrophoresis in the fraction, corresponding to the M-component. Adjacent fractions, which by definition contain background IgG, were practically inactive. IgG, prepared from the sera by ion exchange chromatography, and shown to be rich in M-component by electrophoretic criteria, was highly AST active. In the case of serum AO, one chromatographic fraction contained mostly background IgG and little myeloma globulin; the specific AST activity of this fraction was considerably lower than that of the M-component rich preparations. This finding suggested, that background IgG from serum AO is endowed with a lower specific AST activity than is the case for the corresponding M-component. In this first series of experiments, such information was not obtained for background IgG from the serum EN.

In later reports (Zettervall 1967 a and b) it was given that background gamma globulin of the sera AO and EN showed negligible, or no antibody activity and this was also stated in the case of the ASTA active serum IN. Further, the electrophoretic distribution of the ASTA activity in serum IN displayed a maximum in the M-component region; IgG preparations from serum IN, containing mostly M-component material, proved highly ASTA active.

The present Chapter provides more detailed account on the results sketched in the preceding paragraph and also on some other investigations, aiming at identification and characterisation of the antibody active principle in the sera, AO, EN and IN.

MATERIALS AND METHODS

Serum IN had been obtained from the patient about 8 months before the start of the experiments.

Serum AO; 2 sera, designated serum AO A and B, respectively, were used. Serum AO A had been obtained from a bleeding about 4 years before the experiments, whereas serum AO B was of unknown date; it had probably been stored for at least 2.5 years before use.

Serum EN; 2 sera, EN A and B, were used in the first experiments about 1.5 years after the bleeding. They were obtained from the patient at 5 day intervals. A third serum (EN C) was first titrated 10 months after its collection. The sera may have been accidentally thawed.

Antisera. The following antisera, already described in Chapter I, were used: specific *anti-IgA* and *anti-IgM*; the 2 specific *anti-IgG* sera *A* and *B*; specific *anti-K* and *anti-L*; specific *anti-transferrin* and specific *anti-BSA*. Also used were the sera *anti-IgG AO*, *anti-IgG EN* and *anti-IgG IN*; these sera were absorbed with a mixture of IgG from 2 G myeloma sera of light chain type K and L, respectively; 10 mg of each lyophilised IgG preparation/ml of antiserum was used. At immunodiffusion, absorbed anti-IgG AO, anti-IgG EN and anti-IgG IN still gave precipitin reactions against the respective M-components; no precipitation was noted against normal IgG (Kabi).

Precipitation of lipoproteins from normal rabbit serum and rabbit anti-sera was performed according to Burstein and Samaille (1958).

Inhibition of AST and ASTA activity by antisera was studied by means of the blood agar diffusion techniques, described in Chapter I. Equal volumes of suitable dilutions (0.15 M sodium chloride) of the sera AO, EN or IN and antisera or normal rabbit serum were mixed after which the strepto- or staphylolysin inhibitive power of the mixtures was assayed with the respective plate titration methods as given in Chapter I. When the anti-transferrin or the anti-BSA sera were used, the patient serum dilutions had been mixed with BSA or transferrin to a concentration equal to, or exceeding that of the M-component. When saline solutions, containing transferrin or BSA at this concentration (*i.e.* 0.005 g/100 ml) were mixed with equal volumes of anti-transferrin or anti-BSA sera, respectively, and the mixtures tested against the respective antisera and antigens in immunodiffusion experiments, antibody excess was apparent. The antisera used in the inhibition experiments were, besides anti-transferrin and anti-BSA, anti-IgG A and B, anti-K and anti-L. In mixtures of patient sera and the antisera anti-IgG A, anti-IgG B and anti-K, similar to the mixtures used in the plate experiments, antibody excess was demonstrated at immunodiffusion. The same was true for the mixtures of anti-L and the sera EN and IN; with serum AO as antigen and antibody from anti-L these were approximately at equivalence. The antisera had been delipidised before use. Without this treatment, several antisera showed AST activity. This was negligible after the dextran precipitation. The anti-transferrin serum could not be used in experiments with ASTA material since this antiserum inhibited staphylolysin. Controls employed were: saline, serial dilutions (saline) of patient sera, saline solutions of BSA or transferrin, rabbit sera diluted 1:1 with saline. The control preparations applied to the plates had been made to contain dextran sulphate and calcium chloride at the same concentrations as in the mixtures to be tested.

Immunochemical procedures. Immuno-electrophoresis of the sera AO, EN and IN, employing anti-IgG A, anti-K and anti-L sera was used for classification of the M-components.

The content of light chain antigenic determinants K and L in IgG preparations was assayed in immunodiffusion experiments. Serial dilutions of the preparations were tested against anti-K and anti-L sera and the highest dilutions giving visible precipitates were noted. Knowing initial protein concentrations in the samples the protein concentrations corresponding to these highest dilutions were calculated and will be referred to as final concentrations. For each protein preparation a K/L ratio could then be determined from the definition formula, $K/L \text{ ratio} = (\text{final concentration against anti-L}) / (\text{final concentration against anti-K})$.

IgG content of samples was measured with the single radial immunodiffusion technique, using a suitable dilution of anti-IgG A serum in the plate. Serial dilutions of IgG, freshly obtained from the peak fraction at gel filtration (Sephadex G-200, 0.1 M tris, pH 8.0) of IgG from pooled normal human serum, was employed for calibration. M-component in chromatographic fractions was determined with the same technique by use of the respective rabbit sera anti-IgGAO, EN and IN, absorbed as described above. M-component-rich preparations were used as standard solutions.

Determination of Gm groups in sera and preparations of M-components was performed by the Institute of Medical Microbiology, University of Lund.

Determination of serum total protein concentration was carried out according to the Lowry method. The figures found, based on calibration of the assay system with IgG, were multiplied with a correction factor. This was found in experiments with mixtures of albumin and IgG (Kabi) with ratios of the two proteins equal to those found for the sera at electrophoresis. The protein content of concentrated IgG solutions was determined by the ordinary Lowry method. The values were expressed as optical densities (OD_{280}).

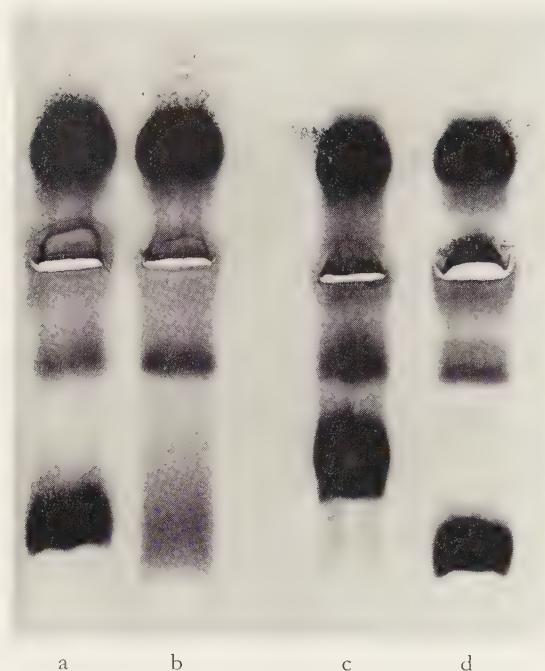


Fig. 1. Agar gel electrophoresis of a) serum IN; b) normal human serum; c) serum AO A; d) serum EN A. The anode is at the top of the figure.

RESULTS.

Case IN.

Electrophoretic and antigenic characteristics of serum and M-component.

Agar gel electrophoresis of serum IN is illustrated in Fig. 1 a, and of simultaneously analysed normal serum in Fig. 1 b. Patient serum is seen to contain an M-component of γ_2 -mobility; background gamma globulin is scanty and this is especially apparent when the pattern is compared with that of the gamma globulin in the normal serum.

Paper electrophoresis of serum IN gave the figures of 2.6 and 0.33 g/100 ml for the concentration of M-component and background IgG, respectively.

Starch gel electrophoresis of serum IN and of purified M-component showed heterogeneity of the M-component in both types of samples. The two patterns appeared similar; at least 3 closely spaced protein bands were discernible in each of the patterns. The spacing of the

bands and their position in the gel was the same for both types of material. However, the relative intensities of the bands differed in that more material seemed to be distributed at the cathodic end of the pattern from the purified M-component than was the case for that from the serum sample. No protein fractions strictly outside the M-component region were observed for the purified material.

Immunoelectrophoresis of serum IN classified the M-component as being of type GL.

The Gm type of serum IN was Gm (1, 2, 4, 5) and that of the M-component was Gm (1, 2-4, -5).

Characterisation of the serum ASTA activity.

Serum ASTA titre was first determined about 8 months after collection of the sample. When the initial dilution of serum at titration was 1:15,000 the figures found in duplicate determinations were 3400 and 3600, mean 3500 ASTA U/ml. With initial dilution 1:10,000 the titres obtained in the same experiment were 3000 and 2600, mean 2800 ASTA U/ml. Heating at 56°C for 30 min. did not appreciably change the titre. Ageing considerably affected the serum titre. Thus titres of about 700 ASTA U/ml were obtained in several aliquots of the serum when titrated about 1 year after the initial experiments, i.e. 1.5 years after collection.

Electrophoretic distribution of the serum ASTA activity was first studied by preparative cellulose acetate electrophoresis of serum IN. Corresponding to the albumin, α_1 , α_2 , β and γ regions, as seen on the stained guide strips, fractions were cut and eluted in a standard volume of phosphate buffered saline (PBS) containing 0.1 per cent (w/v) BSA. ASTA activity was only found in the fraction corresponding to the gamma globulin region. The serum was further investigated by preparative electrophoresis in Sephadex G-25 (Fig. 2); 0.5 ml of serum was fractionated for 13 hrs. and protein was eluted with PBS. Definite ASTA activity was localised in the fractions 20-24. There was a striking parallelism between titres and protein content. Both

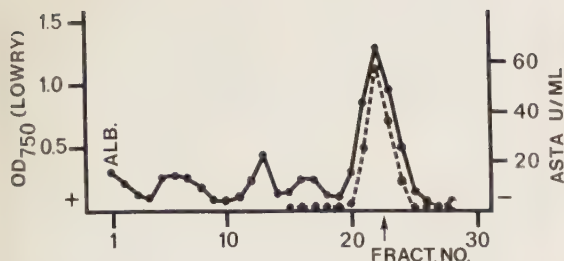


Fig. 2. Preparative electrophoresis of serum IN in Sephadex G-25 block. The arrow denotes the point of application of the serum. The solid line gives the optical density at 750 nm at protein determination according to Lowry. The broken line signifies the ASTA titre (U/ml) of the fractions titrated, *i.e.* 15–28. No ASTA activity was found in the fractions 15–19 and 25–28. *Alb.* signifies the position of the albumin fraction.

achieved their maxima for fraction 22. This eluate, concentrated to a protein content of 0.73 g/100 ml, was analysed by agar gel electrophoresis and by immunodiffusion against anti-IgA and anti-IgM sera. Only M-component was discernible in the electrophoretic pattern and no reaction with the antisera was noted. Since, for instance, fraction 19 gave precipitates against these antisera when analysed at a protein concentration of 0.051 g/100 ml, fraction 22 could not contain more than $0.051/0.73 = 1/14$ of its protein as IgA or IgM. The specific ASTA activity of the protein in fraction 22 was calculated to 52 ASTA U/OD₂₈₀/ml. The serum titre, determined at the start of the preparative electrophoresis was 1480 ASTA U/ml. Calculations based on the definite titres obtained and the volumes of the eluates gave the figure of 96 per cent for recovery of the serum ASTA activity in the eluates. In these calculations the void volume of the Sephadex fractions had been taken in regard, calculated as $1/3$ of the gel volume, determined at centrifugation of the fractions in graded tubes. The void volume thus found amounted to 6 per cent of the buffer volume, used for elution. Taking the figure for the concentration of M-component in serum IN from the paper electrophoretic determination already given, *i.e.* 2.6 g/100 ml,

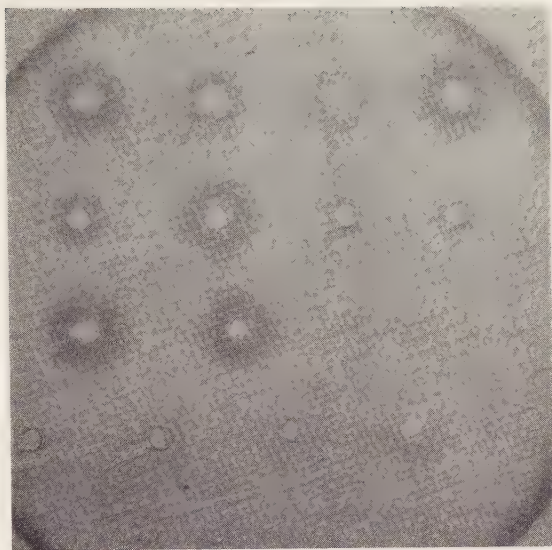


Fig. 3. Inhibition of serum IN ASTA activity by antisera. From left to right, Row 1: serum IN 1:1200, 1:3600, 1:7200, and 1:600+BSA+NaCl. Row 2: serum IN 1:600+anti-L, 1:600+anti-K, 1:600+anti-IgG A and 1:600+anti-IgG B; Row 3: serum IN 1:600+BSA+anti-BSA, 1:600+normal rabbit serum; Row 4: anti-L, anti-K, anti-IgG A, anti-IgG B, anti-BSA, all mixed with NaCl; Row 5: normal rabbit serum+NaCl, and BSA+NaCl+dextran sulphate+CaCl₂.

in conjunction with the specific activity of protein in fraction 22, *i.e.* 52 ASTA U/OD₂₈₀/ml leads to the estimate of the serum titre of 1839 ASTA U/ml. The concentration of M-component in the serum, calculated from the protein content of the M-component peak was 2.5 g/100 ml (100 per cent recovery assumed).

Inhibition of serum IN ASTA activity by antisera (Fig. 3). Serum IN, at a dilution of 1:600 was tested. BSA-anti-BSA was used for the unspecific precipitate control. When normal rabbit serum, or anti-K was added to serum IN, no decrease in the diameter of the haemolysis inhibition zones was apparent in comparison with the appropriate control preparations. A slight decrease in zone diameter was sometimes obtained when anti-BSA serum was added to the mixture of BSA and serum IN. A nearly complete extinction of the haemolysis inhibition zone was noted with anti-IgG

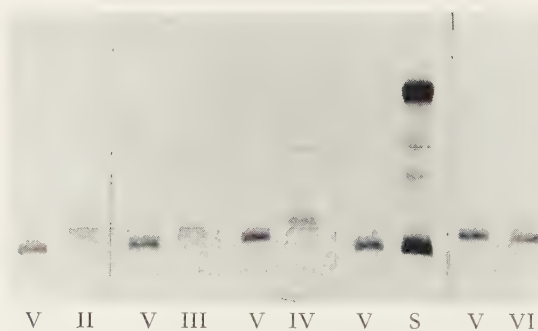


Fig. 4. Agar gel electrophoresis of the chromatographic fractions IgG IN II—VI from serum IN and of the diluted (1 : 4) serum. Roman numerals indicate the fractions, and S the serum. The anode is at the top of the figure.

A and anti-IgG B. A marked inhibition was given by the anti-L serum. This inhibition was always greater than that from BSA-anti-BSA. In addition to the lysis outside the inhibition zones there was also lysis around the central wells. In the cases the inhibition zone diameter was decreased also the central lysis was impaired. BSA, normal rabbit serum, the rabbit antisera and saline, gave no significant haemolysis inhibition.

Preparation, characterisation and specific ASTA activities of IgG from serum IN.

IgG fractions, containing various amounts of background gamma globulin and M-component were obtained by ion exchange chromatography; 15 ml of serum, dialysed against 0.0175 M, pH 6.50 phosphate, was chromatographed in DEAE-Sephadex A-50 equilibrated with the same buffer. The buffer was also used for elution. The protein emerged as one peak. The major part of the eluate was fractionated in CM-Sephadex C-50 "Fine" equilibrated and initially eluted, with the mentioned phosphate buffer. Subsequently the eluent was changed to: 0.0175 M phosphate — 0.02 M sodium chloride, pH 6.40; 0.0175 M phosphate — 0.03 M sodium chloride, pH 6.46, and from this buffer in a gradient procedure towards 0.5 M sodium chloride as limit buffer. The fractions obtained during the stepwise

and in the first part of the gradient elution procedures contained little protein. Pools of fractions were taken from this part of the chromatogram and are designated as IgG IN I—IV, where numbering denotes order of elution. Later during the gradient elution a major protein peak emerged. This material was collected in 2 pools of which IgG IN V contained most of the protein. IgG IN VI which was taken from the descending part of the peak, was poor in protein. Flow rate was approximately 30 ml/hr in both the DEAE- and the CM-Sephadex chromatography and column dimensions were 2×20 cm.

If necessary the pools were concentrated by ultrafiltration for the analyses to be described below.

Agar gel electrophoresis (Fig. 4) was performed on aliquots of the pools, dialysed against distilled water and further concentrated by lyophilisation. However, IgG IN I contained too little protein and was not analysed. The dried protein was dissolved in PBS so as to give solutions of the calculated protein concentration of 0.35 g/100 ml. Included in the analysis was serum IN diluted 1 : 4 with PBS. IgG IN V contained mostly M-component as shown by homogeneity of the protein and the coincidence of its electrophoretic mobility with that of the M-component from the serum sample. IgG II—IV contained heterogenous protein mostly migrating at the anodic side of the M-component. IgG VI gave the picture of a band with the approximate mobility of the M-component; in addition, a diffuse portion extending in the direction of the cathode was evident.

Immunochemical analysis of the fractions IgG IN I—VI was carried out with immunodiffusion against anti-K and anti-L, and for IgG I, II, III and IV in addition with anti-IgA and anti-IgM sera. Further, several fractions were analysed by single radial immunodiffusion employing anti-IgG A and absorbed anti-IgG IN sera, respectively.

Immunodiffusion of IgG IN V at a concentration of 0.68 g/100 ml against anti-IgA and anti-IgM sera gave no precipitates, which,

however, was the case when normal human serum at 1 : 10 dilution was similarly investigated. No reaction was seen with IgG IN I, II and III at the concentrations (g/100 ml) 0.04, 0.07 and 0.18, respectively.

The L/K ratios are given in Table 1. Also shown are the results of the single radial immunodiffusion experiments with anti-IgG A and absorbed anti-IgG IN serum given as weight per cent of IgG based on the sample's total protein. With anti-IgG A serum M-component evidently gives spuriously high values as seen for IgG IN V. Using absorbed anti-IgG IN serum in the plate the figures for contamination with M-component are probably overestimated since admixture of polyclonal IgG (Kabi) to IgG IN V lead to an increase of precipitin ring diameters compared to that from pure IgG IN V. No ring was obtained with IgG Kabi alone or with IgG IN I and II.

Specific ASTA activities of protein in the fractions IgG IN I—VI as calculated from titre and protein concentration figures for the samples are found in Table 1. The unfractionated serum IN had the titre, 1200 ASTA U/ml, which corresponds to a calculated specific ASTA activity of the M-component in serum of 34 ASTA U/OD₂₈₀/ml. For the IgG fractions there is evident a rough positive correlation between specific ASTA activity on one hand and L/K ratio, as well as percentage figures from the single radial immunodiffusion experiments on the other. The maximum specific activity (17 ASTA U/OD₂₈₀/ml) was obtained for fraction IgG IN V which also gave the maximum figures for the L/K ratio and for

the percentages obtained from both types of single radial immunodiffusion experiments. Since the anti-K serum detected type GK immunoglobulin in IgG Kabi, IgG IN II and III at about 0.01 g/100 ml but not in IgG V at 0.17 g/100 ml, the content of background IgG in IgG IN V could be estimated to $<0.01/0.17 = 5.9$ per cent. On the other hand, considering the single radial immunodiffusion data for IgG IN II, it was calculated that practically 100 per cent of the total protein content of this fraction consisted of non-M-component IgG. Thus the figure for specific ASTA activity of IgG IN II material, <1.5 ASTA U/OD₂₈₀/ml, is practically the same as could be calculated for the background IgG in the sample. In the calculation, the figure for the IgG content of fraction IgG IN II as obtained with the anti-IgG A serum has been subtracted with 2 times the value for the fraction's M-component content, as found with the anti-IgG IN serum. (The anti-IgG A serum overestimated M-component by 100 per cent as seen with IgG IN V.) Similar calculations for IgG IN III gave at least 78 per cent background IgG of the total protein and a specific activity of that IgG of at most 1.7 ASTA U/OD₂₈₀/ml.

Case AO.

Electrophoretic and antigenic characterisation of serum and M-component.

Agar gel electrophoresis of serum AO A demonstrated the presence of a M-component of γ_1 mobility; background IgG of relatively low concentration was seen at the cathodic side of the band protein (Fig. 1 c).

Table 1. *Immunochemical and serological characteristics of IgG fractions from serum IN*

Material IgG IN	I	II	III	IV	V	VI
L/K	<0.50	0.25	0.50	1.0	>32	2.0
IgG % w/w	46	105	110		200	175
M-component % w/w	<7	<3.5	16	50	100	50
Specific ASTA activity U/OD ₂₈₀ /ml	<2	<1.5	<1.3	6.6	17	9.1

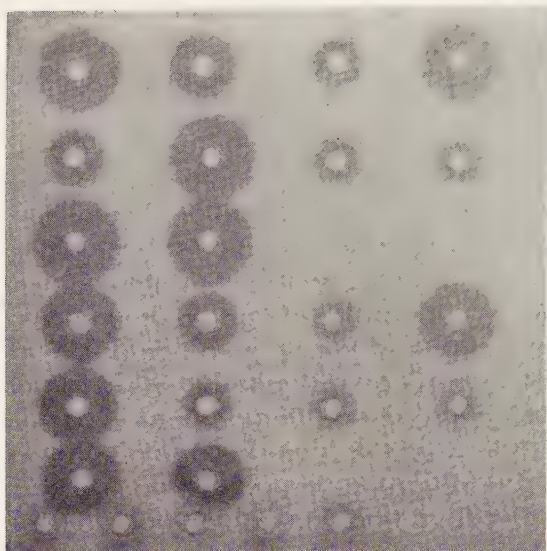


Fig. 5. Inhibition of a) serum AO and b) serum EN AST activity by antisera. From left to right, a) Row 1: serum AO 1:2000, 1:6000, 1:18 000, and 1:1000 + transferrin+NaCl; Row 2: serum AO 1:1000 + anti-L, 1:1000+anti-K, 1:1000+anti-IgG A and 1:1000+anti-IgG B; row 3: serum AO 1:1000+normal rabbit serum, 1:1000+transferrin+anti-transferrin.

b) Row 4: serum EN 1:2000, 1:6000, 1:18 000, 1:1000+transferrin+NaCl; Row 5: serum EN 1:1000+anti-L, 1:1000+anti-K, 1:1000+anti-IgG A, and 1:1000+anti-IgG B; row 6: serum EN 1:1000+normal rabbit serum, 1:1000+transferrin+anti-transferrin; Row 7: anti-L, anti-K, anti-IgG A, anti-transferrin, normal rabbit serum, NaCl+dextran sulphate+CaCl₂, all mixed with NaCl, and transferrin+NaCl+dextran sulphate+CaCl₂. Anti-IgG B+NaCl gave no significant inhibition of haemolysis when tried in another experiment.

Paper electrophoretic quantitation of M-component and background gamma globulin in serum AO A gave the figures of 5.0 and 0.13 g/100 ml, respectively.

Starch gel electrophoresis of serum AO together with purified M-component demonstrated heterogeneity within the myeloma globulin both in the serum and in the purified material. At least 3 bands in close proximity to each other and with the same characteristic spacing and position in the gel were observed in both materials. In addition, the IgG preparation gave rise to a weak and

more cathodic band, not seen in the serum pattern.

Immunoelectrophoresis of serum AO A with anti-K, anti-L and anti-IgG A sera classified the M-component as a GL immunoglobulin. The Gm type of serum and M-component was Gm (1, -2, 4, 5) and Gm (1, -2, -4, -5), respectively.

Characterisation of the serum AST activity.

Serum AST titre. Serum A was titrated in duplicate giving the figures in AST U/ml of 252,000 and 363,000, mean 308,000. Serum AO B gave the titre of 230,000 after a first thawing. Thousandfold dilutions of this serum in PBS were stored frozen. When titrated 5 months later, the titre obtained was 240,000 AST U/ml of undiluted serum. When serum B was dialysed in the cold for 30 hr against 0.0175 M, pH 6.5 phosphate the titre dropped to 100,000 AST U/ml. The change in protein concentration and volume of the serum at dialysis was insignificant.

Serum AO A was heated at 56°C for 30 min. and titrated in duplicate. A considerable drop in titre from 308,000 AST U/ml for untreated serum to 59,000 and 73,000, mean 66,000 AST U/ml was noted.

Inhibition of serum AO B AST activity by antisera (Fig. 5). The serum was used at a dilution of 1:1000. Transferrin-anti-transferrin was used for the unspecific precipitation control. Almost complete suppression of the haemolysis inhibition zone on the blood agar streptolysin plate was obtained with the anti-IgG A and B sera. Partial suppression was obvious with the anti-L serum. No inhibition was discernible with the normal rabbit, the anti-K or the anti-transferrin sera. The controls without patient serum, i.e. normal rabbit serum, the antisera, transferrin and saline gave no significant inhibition of haemolysis.

Preparation, characterisation and specific AST activities of IgG from serum AO A.

Approximately 15 ml of serum AO A,

dialysed against 0.0175 M, pH 6.50 phosphate, was chromatographed on a 2×22 cm column of DEAE-Sephadex, equilibrated and eluted with the same buffer. Protein emerged in one peak. The eluate was saved. This preparation is referred to as IgG AO I. Serum proteins, remaining on the column, were eluted with 0.175 M phosphate, pH 6.50. This eluate was concentrated, equilibrated with 0.0175 M, pH 6.50 phosphate and applied on a 2×22 cm column of DEAE-Cellulose (Serva), equilibrated with such buffer. The same buffer was used as eluent; flow rate in both chromatographies was approx. 35 ml/hr. Protein (IgG AO II) was eluted in one peak. The material retained on the column was discarded. IgG AO I and II were analysed as described below. If necessary, the fractions were concentrated by ultrafiltration.

Agar gel electrophoresis of IgG AO I and II was carried out at a protein concentration of 0.35 g/100 ml; serum AO A, diluted 1 : 4 with PBS was also analysed. The IgG samples had been made up to the correct protein concentration by addition of 3 volumes of PBS. As seen from Fig. 6, IgG AO I contained electrophoretically heterogeneous protein migrating mostly at the cathodic side of the obvious M-component in IgG AO II. In IgG AO II a faint protein band was found at the anodal side of the predominating M-component.

Immunochemical analysis of IgG AO I and II was carried out with the same technique as used for characterisation of IgG IN. However, absorbed anti-IgG AO serum was used in one of the single radial immunodiffusion experiments. When analysed at the protein concentrations of 0.7 and 0.55 g/100 ml against anti-IgA and anti-IgM sera, IgG AO I and II respectively gave no precipitates. The 2 antisera gave precipitates against normal human serum diluted 1 : 5.

AST titration of IgG AO I and II gave the specific AST activity figures of <1.3 and 1400 AST U/OD₂₈₀/ml, respectively. The L/K ratios and the results of the single radial immunodiffusion experiments as well as the specific activities found are listed in Table 2.



Fig. 6. Agar gel electrophoresis of the chromatographic fractions IgG AO I and II from serum AO and diluted (1 : 4) serum. Roman numerals indicate the fractions and S the serum. The anode is at the top of the figure.

Judging from the single radial immunodiffusion data, IgG AO I consisted of practically pure background IgG, which agreed with the electrophoretic picture of the protein and the absence of reaction against anti-IgA and anti-IgM sera in immunodiffusion. Thus the low specific AST activity for IgG I material, *i.e.* <1.3 AST U/OD₂₈₀/ml was also considered to apply to the serum AO A background IgG in the sample. The results of the immunodiffusion experiments with the anti-IgA and anti-IgM as well as the electrophoretic findings demonstrated only M-component in IgG AO II

Table 2. *Immunochemical and serological characteristics of IgG from serum AO*

Material IgG AO	I	II
L/K	0.25	8.0
IgG % w/w	114	34
M-component % w/w	<3	100
Specific AST activity U/OD ₂₈₀ /ml	<1.3	1400

besides the quantitatively unimportant protein fraction seen at electrophoresis. Calculating from the final concentrations at immunodiffusion of both IgG preparations against anti-K serum, the contamination of IgG AO II with background IgG was estimated as $0.009/0.08 = 11$ per cent. Hence most of the protein of IgG AO II seemed to consist of M-component. The figure found for the specific activity of IgG AO II, *i.e.* 1400 AST U/OD₂₈₀/ml based on the total protein concentration was thus approximately the same as could formally be assigned to the M-component. Calculations based on the serum AST titre (308,000 AST U/ml) and the concentration of M-component in the serum (5.0 g/100 ml) lead to a formal specific AST activity of 4500 AST U/OD₂₈₀/ml for the myeloma globulin.

Case EN.

Electrophoretic and antigenic characteristics of serum and M-component.

Agar gel electrophoresis of serum EN A demonstrated the presence of an M-component of γ_2 mobility (Fig. 1 d). Very little background gamma globulin seemed to be present.

Paper electrophoretic quantitation of M-component and background gamma globulin in serum EN A gave as result 3.8 and 0.07 g/100 ml, respectively.

Starch gel electrophoresis of serum EN A together with purified M-component demonstrated for both preparations a pattern of at least 3 protein bands. Comparison between the patterns showed the same characteristic spacing and position in the gel of the bands.

Immuno-electrophoresis of serum EN A classified the M-component as a GK protein.

Gm groups in serum and M-component EN. The types found were: for serum Gm (—1, —2, 4, 5) and for purified M-component Gm (—1, —2, 4, —5).

Characterisation of the serum AST activity.

Serum AST titre. Repeated titrations on aliquots of serum EN A gave titre values between 49,000 and 105,000. The lastmentioned figure

was obtained in an experiment where a duplicate sample gave the titre of 65,000. The discrepancy was thought to be due to a prozonal phenomenon (*cf.* Chapter V). Serum EN B, gave the titre figure of 113 000 U/ml. Serum EN C, titrated in duplicate showed a titre of 104,000—100,000, mean 102,000 U/ml.

In the last experiment serum EN C was also titrated after heating at 56°C for 30 min. giving titres in duplicate determinations of 34,000 and 37,000, mean 35,500 AST U/ml. Hence, heat treatment reduced the serum AST activity to about 1/3 of that of untreated serum.

Serum EN A was dialysed for 30 hr in the cold against 0.0175 M phosphate, pH 6.5 while another aliquot of the same serum sample stood at the same temperature for the same time. An insignificant precipitate formed during dialysis. The change in total protein concentration of the dialysed serum as obtained by comparison with the protein content of the control sample was therefore taken as a measure of volume change at dialysis. When corrected for the volume change the titre of the dialysed serum nearly coincided with that of the control sample, *viz.* 60,500 and 60,600, respectively (means of duplicate determinations).

Inhibition of serum AST activity by antisera (Fig. 5). The experiments were set up as has been described for similar investigations on serum AO. The results were: definite inhibition by anti-K, and almost complete inhibition by the 2 anti-IgG sera. No inhibition was noted with normal rabbit serum, transferrin-anti-transferrin or anti-L. The controls without serum EN, *i.e.* the rabbit sera, transferrin and saline gave no definite inhibition of haemolysis.

Preparation, characterisation and specific AST activities of IgG from serum EN.

A pool of serum A and B was dialysed against 0.0175 M, pH 6.50 phosphate. Of the serum 15 ml was chromatographed on DEAE-Sephadex A-50. The column (2 × 20 cm) had been equilibrated with the same buffer which

was also used for elution (flow rate approx. 35 ml/hr). Protein emerged in one peak. Most of the eluate was applied on a 2×20 cm column of CM-Sephadex C-50 "Fine", equilibrated with the same buffer. This, *i.e.* 0.0175 M, pH 6.50 phosphate, was initially used for elution. The procedure was continued in 2 steps with 0.0175 M phosphate — 0.03 M sodium chloride, pH 6.46 and 0.0175 M phosphate — 0.04 M sodium phosphate, pH 6.42, respectively. Finally, gradient elution was carried through from the last-mentioned buffer to 0.5 M sodium chloride as limit buffer. The flow rate was approx. 30 ml/hr.

Little protein was obtained in the eluates at the stepwise and in the beginning of the gradient elution. The pools of fractions from this part of the chromatogram are denoted IgG EN I—III. Following the protein poor fractions a sharp peak, containing most of the eluted material, came off the column. The pool of fractions from the ascending part of the peak was designated as IgG EN IV, that from the middle of the peak as IgG EN V and the pool from the descending part as IgG EN VI. IgG I contained very little protein, and was discarded whereas IgG II—VI were further analysed after concentration by ultrafiltration.

Agar gel electrophoresis of the samples IgG II—IgG VI. Aliquots of the samples were dialysed against distilled water and lyophilised. Protein was then dissolved in 0.15 M sodium chloride to a calculated protein concentration of 0.35 g/100 ml and submitted to agar gel electrophoresis. Serum EN A, diluted 1:4 with saline, was used as a reference. As seen from Fig. 7, IgG EN V contains protein migrating

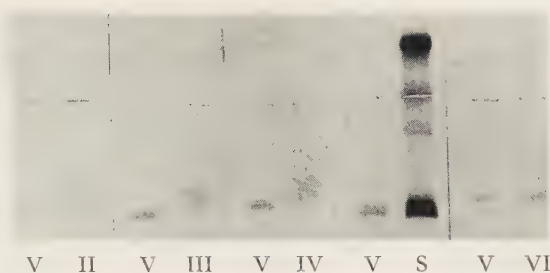


Fig. 7. Agar gel electrophoresis of the chromatographic fractions IgG EN II—VI from serum EN and of diluted (1:4) serum. Roman numerals indicate the fractions and S the serum. The anode is at the top of the Figure.

as the serum M-component. IgG EN II, III and IV are seen to contain electrophoretically diffuse protein with a mean mobility higher than that of the M-component. On the original gels a faint protein stain extending into the M-component region was obvious for IgG III and IV. IgG VI definitely contained M-component and in addition some protein with a higher cathodal mobility.

Immunochemical analysis of the fractions IgG EN II—VI was carried out as for IgG AO and IgG IN, *i.e.* immunodiffusion against anti-K and anti-L sera and single radial immunodiffusion against anti-IgG A and absorbed anti-IgG EN sera, respectively. IgG II, III and V were analysed in immunodiffusion against anti-IgA and anti-Ig M sera.

AST-titration was performed on the preparations IgG EN II—VI.

IgG EN II, III and V, analysed against anti-IgA and anti-IgM sera at a protein concentration of 0.04, 0.09 and 0.6 g/100 ml

Table 3. *Immunochemical and serological characteristics of IgG from serum EN*

Material IgG EN	II	III	IV	V	VI
K/L	2.7	2.0	4.0	>32	8.0
IgG % w/w	71	110	104	145	130
M-component % w/w	<13	31	52	100	67
Specific AST activity					
U/OD ₂₈₀ /ml	<5.8	<5.6	25	680	32

gave no precipitates, which, however, was the case with normal human serum diluted 1 : 10.

Table 3 summarises the findings at immuno- and single radial immunodiffusion as well as the specific AST activities of the IgG EN preparations. In the single radial immunodiffusion experiments with anti-IgG A serum presence of M-component, as in IgG EN V, evidently lead to overestimation of IgG. The values of M-component in IgG EN II, III, IV and VI found with anti-IgG EN serum were probably unduly high. It was shown with the same antiserum that presence of polyclonal IgG (Kabi) expanded the ring from IgG EN V in mixtures of the 2 kinds of protein preparations. IgG Kabi alone gave no visible reaction. As seen from the Table there was co-variation of K/L ratios, estimates of M-component and overshoot of IgG percentage on one hand, and specific AST activities on the other. For IgG EN V the calculated contamination with background IgG was $<0.0184/0.147 = 12.5$ per cent; In the calculation the final concentrations of IgG EN III and V against anti-L have been used. In conclusion, there was no sign of background IgG, IgA or IgM contaminating the M-component in IgG V. The specific AST activity of IgG EN V, 680 AST U/OD₂₈₀/ml was therefore taken as the value of the specific AST activity that could formally be assigned to the M-component in IgG EN V.

The specific AST activity of the M-component in serum EN A, taking the concentration of the M-component (3.8 g/100 ml) from the paper electrophoretic data together with the serum titre (105,000) could be calculated as 2000 AST U/OD₂₈₀/ml.

The amount of M-component in IgG EN II was minor, judged from the result of the single radial immunodiffusion experiment with anti-IgG EN serum. Hence, the percentage of IgG in the preparation, as found with anti-IgG A serum, was considered essentially correct. Therefore the specific AST activity of background IgG in the preparation was calculated as $<5.8/(71 \text{ per cent}) = 8.2$ AST U/OD₂₈₀/ml. Similar calculations for IgG EN III resulted in

the estimate <8.6 AST U/OD₂₈₀/ml for the specific activity of background IgG.

DISCUSSION.

The decrease in haemolysis inhibition zone diameters, which was noted with some of the antisera employed in the blood agar lysin plate experiments with the patient sera AO, EN and IN is explained by one or both of 2 mechanisms. Either the diffusibility or the anti-lysin activity of the serologically active principle of the patient sera is impaired. In the control experiments immunoprecipitation *per se* or normal rabbit serum displayed a minor effect, or none at all. Hence, the markedly inhibitive effect of the anti-IgG, anti-K or the anti-L sera is probably specific. In its turn, this probably implies that the AST or ASTA active principle in the patient sera is bound by the respective rabbit anti-bodies. Such binding should affect the diffusibility of the antilysin principle and should therefore lead to a decrease of the haemolysis inhibition zone diameter irrespective of whether the rabbit antibody impairs the anti-lytic property or not. Hence, the almost complete suppression of the haemolysis inhibition zone diameters from the sera AO, EN and IN with the anti-IgG sera suggests that all, or almost all of the antibody active principle in the patient sera consists of IgG. The inhibition noted with the antisera against the light chain antigenic type of the respective M-components, and the lack of effect with the antiserum against the other light chain type suggests that the serologically active IgG is of the M-component's light chain type. Earlier findings (Zettervall et al. 1966) and the present results from the preparative electrophoresis experiments substantiated that IgG accounts for practically all of the serological activity of the patient sera. In these experiments the serological activity was almost quantitatively recovered from the fractions in the electrophoretic gamma globulin region which represents the bulk of the serum IgG. The electrophoretic distribution of the antibody activity

inside the gamma globulin region strongly suggests that the M-components are the carriers of the activity. This receives ample support from the present findings with IgG preparations from the sera. Thus there is co-variation between indicators of M-component in the preparations on one hand and the specific antibody activity of the samples on the other. The higher the deviation in K/L or L/K ratio from that of polyclonal IgG or the higher the concentration of M-component as found with the anti-individual IgG rabbit sera, the larger the specific AST or ASTA activities. Also in the case of EN and IN the overestimation of IgG with the anti-IgG A serum, an effect which was seen to prevail in the M-components, correlates with the specific serologic activities.

In all 3 cases background IgG samples of comparatively low specific antibody activity have been obtained. This specific activity amounts to less than 1.5 per cent of that of the M-component in the case of EN, less than 1 and less than 10 per cent in the cases of AO and IN, respectively. For polyclonal AST it has been shown (Hedberg and Moritz 1960) that IgG from various chromatographic fractions of individual human sera displays a constant specific AST activity irrespective of the electrophoretic mobility of the protein. It is thus probable for the IgG preparations from the sera AO and EN that the specific activities found for the background IgG are representative for all the polyclonal IgG eluted from the columns. Thus the M-components AO and EN seem to account for the major part of the serological activity in the chromatographic fractions. In fact it could be calculated from the specific activity values found that more than 98.5 per cent of the AST activity in the prepared IgG should belong to the M-components. If the findings for the background IgG specific activity in the case of IN are regarded as typical, the M-component is calculated to account for more than 90 per cent of the ASTA activity in the preparations. Hedberg and Moritz also found coincidence between the calculated

specific AST activity of IgG in the sera and that actually observed in their IgG preparations. These findings may suggest that the low specific activity figures found for the background IgG preparations of AO and EN also reflect a negligible specific AST activity of the native background IgG in the sera. Since the value of the specific AST activity of background IgG from serum AO (< 1.3 U/OD₂₈₀/ml) amounts to less than 1/5 of that of the pooled normal IgG (6.5 U/OD₂₈₀/ml) the background IgG may be of lower specific activity than is normally seen in polyclonal AST active IgG. Whether this also may apply to other antibody specificities in the 2 kinds of polyclonal IgG preparations is at present unknown. Presence of M-component in IgG EN III, IV and VI was demonstrated with the anti-IgG EN serum. Calculating from these values and the calculated specific AST activity of the M-component in IgG EN V, the specific AST activities of IgG EN III, IV and VI become much higher than those actually observed. The same type of argument holds for IgG IN III. It may be that overestimation of M-component by the single radial immunodiffusion method is excessive, or that M-component material in the fractions IgG EN III, IV and VI and in IgG IN III is of lower specific activity than that of IgG EN V and IN V, respectively. Earlier experiments (Zettervall 1967 b) indicating AST activity in several of the subfractions of the heterogenous M-components failed to evaluate the differences in specific AST activity, if any, between the various components seen at starch gel electrophoresis.

The specific activities of the M-components from fractions IgG AO II, IgG EN V and IgG IN V lie far below the values calculated from titres and concentrations of M-component in the corresponding sera. The ratios between the figure from serum and that for purified material is about 3 for AO and EN and 2 for IN. In the last case the ratio is even higher if the calculations are based on the titres of serum IN less aged than that used as source of IgG IN V. It is not likely, that an

error in the paper electrophoretic quantitation technique is the explanation especially since the figure for the concentration of the M-component obtained from the preparative electrophoresis of serum IN (2.5 g/100 ml) nearly coincides with that of the other method (2.6 g/100 ml). As recovery of serological activity from the Sephadex block was nearly complete, the same should apply for protein in general, *i.e.* for the M-component. Several other possible explanations are at hand, *e.g.* (i) damage of M-component at preparation (denaturation), (ii) loss of activity present in native background IgG, (iii) selective loss of AST active material, whether mono- or polyclonal, during preparation, or (iv) error in titre values due to a prozonal phenomenon.

Hypothesis (i) is difficult to evaluate. At least from gel filtration data it seems unlikely, that aggregation could play a major role (cf. Chapter IV). (ii) This explanation is improbable in the light of Hedberg and Moritz' finding of equality between specific activities of purified polyclonal AST active IgG and that calculated for the serum gamma globulin. (iii). A mechanism, resulting in selective loss of antibody active material is hard to envisage for polyclonal AST active IgG. The chances may be greater for M-component material, since its heterogeneity is less than that of background IgG; the selective loss of one very active sub-fraction may severely affect the specific activity of the preparation. The differences seen in the relative intensities of the M-component sub-bands, when serum and purified material are compared at starch gel electrophoresis, may be of importance in this context. (iv). This topic is discussed in Chapter V.

The results at agar gel electrophoresis of sera AO and EN show that AST active M-components of widely different electrophoretic mobilities occur (the M-component EN has earlier (Zettervall et al 1966) erroneously been described as of γ_1 instead as of γ_2 mobility). The M-components AO and EN are of different light chain antigenic types and the same applies to the G myeloma globulins of the 2 sera with excessive AST activity

recently described by Kronvall (1967). Thus the situation existing for IgM M-components in cases of the chronic cold agglutinin syndrome is not prevailing for the IgG M-components with AST activity. As is known such cold agglutinin active M-components belong exclusively, or almost exclusively to one light chain antigenic type, *i.e.* K (Franklin et al 1963, Harboe and Deverill 1964).

There is a known strict relationship between Gm and heavy chain subgroup antigens (Kunkel, *et al.* 1964), in that each Gm antigen is strictly limited to only one subgroup. In the case of the M-components discussed in this work all 3 are found from their Gm types to belong to the subgroup IgG 1. *A priori*, this is not an unexpected finding, since about 70 per cent of G-myeloma globulins belong to this subgroup (Grey and Kunkel 1964, Terry and Fahey 1964).

SUMMARY

The M-component from the very high titred ASTA active serum IN and the highly AST active sera AO and EN is shown to contain antibody activity. Antibody activity was not demonstrated in preparations of background IgG from the sera with the techniques employed. If present the corresponding specific antibody activity is of the order of 1 per cent of that of the M-component for cases AO and EN, at most 10 per cent for the case IN. The specific serological activity of the purified M-components is considerably lower, than that calculated from titre and concentration of M-component in the sera. The AST activity of sera AO and EN is heat labile whereas the ASTA activity of serum IN is not. The serum IN activity is more sensitive to ageing than in the case of AO and EN. The AST activity of serum AO is sensitive to exposure to low ionic strength phosphate buffer. The Gm types of the 2 AST active M-components differ as do their antigenic light chain types. All 3 M-components belong to the same heavy chain subgroup, IgG 1.

III. Properties of the papain fragments of IgG AO, EN and IN

The work of Porter (1959) on rabbit gamma globulin showed that papain cleaves the IgG molecule into 3 fragments of approx. the same size. Each of the 2 identical Fab fragments contains a heavy chain fragment (Fd) and most if not all of a light chain from the parent IgG molecule (Fleischman et al. 1963). The third fragment, *i.e.* the Fc fragment consists of the remaining halves of the heavy chain not present in the Fab fragment. The Fc fragment is devoid of light chain material. If the gamma globulin contains antibody activity only its Fab fragment retains the power to combine with the antigen, after papain splitting of the IgG molecule. The Fc fragment shows no antibody property (Porter *op. cit.*). Papain splits IgG from other species, among them man, in principally the same way (Hsiao & Putnam 1961, Fougereau and Edelman 1965). Antibody activity has also been demonstrated in the Fab fragment of human IgG (Tan and Epstein 1963).

AST activity has been associated with the Fab fragment of IgG from sera with extremely high AST titres and IgG M-components (Kronvall 1967, Zettervall 1967 a) among them from the cases AO and EN. The same pertains to Fab from IgG IN (last ref.). The Fc fragments from IgG AO, EN and IN seemed devoid of antibody activity.

Since the investigations on the papain fragments of IgG AO, EN and IN were only qualitative an extended study of their properties seemed warranted. This Chapter provides quantitative data on preparations of papain fragments from the 3 M-components.

MATERIALS AND METHODS

Preparation of IgG: AO; The preparation IgG AO II was described in Chapter II.

EN; 5 ml of serum EN A, dialysed against 0.0175 M, pH 6.50 phosphate was chromatographed on a 1×20 cm column of DEAE-Sephadex A-50, equilibrated and eluted with the same buffer. Flow rate was approx. 5 ml/hr.

IN; 5 ml of serum IN was separated with the same technique. When analysed at a protein concentration of 0.75 g/100 ml by agar gel electrophoresis the proteins appeared as pure M-components.

Preparation of light chains. The same preparation from normal human IgG as described in Chapter IV was used.

Determination of protein concentration. Chromatographic fractions were assayed for protein by spectrophotometry at 280 nm. All other protein determinations were carried out with the Lowry method.

Agar gel electrophoresis. Protein samples in 0.0175 M, pH 6.50 phosphate buffer were mixed with 1/20th volume of 2 M tris-HCl buffer, pH 8.0 before electrophoresis. The addition was made in order to prevent distortion of the electrophoretic pattern which otherwise was apparent.

Antisera. The following antisera, already described in Chapter I, were used in immunoelectrophoresis and in inhibition experiments: antiserum against normal human IgG, absorbed with normal human light chains (*anti-IgG A*), antiserum against normal human heavy chains, absorbed with light chains (*anti- γ -chain*), antiserum against normal human light chains, absorbed with heavy chains (*anti-light chain*), antisera against IgG from sera AO, EN and IN (*anti-IgG AO*, *anti-IgG EN* and *anti-IgG IN*). All sera used in the antibody inhibition experiments were dextran precipitated.

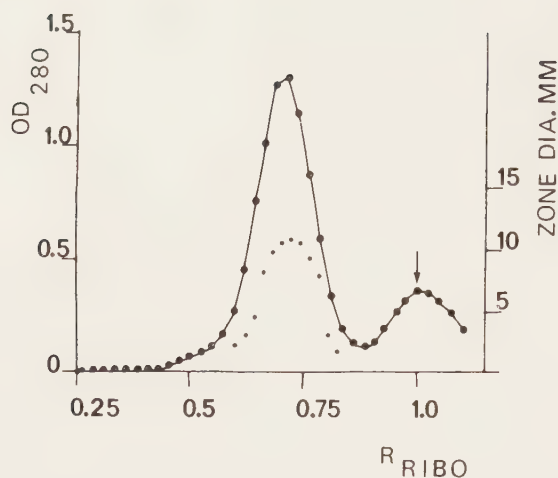


Fig. 1. Gel filtration in Sephadex G-200-phosphate buffered saline of papain digest of IgG AO and subsequent chromatography in the same column of fraction AO D from preparative electrophoresis. First experiment: OD₂₈₀ (ordinate left, full line) of fractions. Second experiment: Haemolysis zone inhibition, diameters in mm (ordinate right, dots) at titration of the fractions in streptolysin blood-agar diffusion plate. The abscissa gives the elution volumes relative to that of riboflavin. Arrow denotes the riboflavin peak.

Immunoelectrophoresis was mostly performed as described in Chapter I. Sometimes a comparative method (Osserman 1960, Wadsworth and Hanson 1960) was used.

Papain digestion of IgG. Solutions of IgG in 0.0175 M, pH 6.50 phosphate buffer and containing 15 mg protein per ml were mixed with 1/10 volume of 0.02 M ethylenediaminetetraacetic acid, disodium salt (EDTA- Na_2) and 1/10 volume of 0.01 M cysteine, both in the mentioned phosphate buffer. Mercuripapain (Worthington Biochemical Corp., Freehold, New Jersey, USA; batch No. 6113) contained in less than 1/20 volume of ethanol was then added. The protein to enzyme ratio was 1 : 100 (w/w). Digestion was carried out at 37°C for periods as described below. Digestion was stopped by addition of 1 original protein sample volume of 0.001 M para-chloromercuribenzoate in phosphate buffer. Control samples of IgG, EDTA- Na_2 and cysteine in the same proportions as used

in the digestion mixtures but without papain were prepared. They were incubated and then mixed with para-chloromercuribenzoate as was the case with the mixtures containing papain.

RESULTS.

Action of papain on the M-components. Papain digestion of the M-components was carried out for various time periods. The digests were analysed by agar gel electrophoresis together with control IgG samples. If papain was allowed to act for a sufficient time the digests were seen to contain a fast and a slow electrophoretic component indicating splitting of the IgG. The susceptibility to papain action was markedly different for the 3 M-components. IgG AO was very rapidly degraded. Thus after 10 minutes digestion the fast and slow components were present but undigested IgG could not be detected. Digestion for longer periods reduced the intensity of both electrophoretic components and the fast fragment was barely visible after a half hours papain treatment. The protein IN was more resistant and little cleavage seemed to have occurred after 10 min. After 30 min. however, only traces of undigested IgG was discernible whence strong fast and slow components were apparent. Further digestion slowly reduced the intensity of both components. IgG EN was the least papain sensitive. Fast and slow components were barely seen after 10 min. digestion. After 1 hr. much of the M-component remained but was virtually absent after 4 hrs. At this time much of the fragments were still present.

Preparation of papain fragments. IgG AO, IgG EN and IgG IN were digested for 10, 30 and 240 min., respectively. The samples, originally containing 30–45 mg IgG, were concentrated to approx. 1.2 ml, mixed with riboflavin and gel filtered in Sephadex G-200 equilibrated with PBS under the conditions described in Chapter I. Column dimensions were 0.9–1.1 × 90 cm. The result is illustrated in Figure 1. In all 3 cases a major protein peak with its

maximum at $R_{\text{ribo}} 0.715-0.740$ was evident.

The peaks were preceded by insignificant shoulders in the region of IgG ($R_{\text{ribo}} 0.550-0.575$), as determined by gel filtration of radioiodinated M-components (Chapter IV). Pools of fractions were collected from the central parts of the major peaks.

The pools were concentrated to 0.5–1 ml by ultrafiltration. Most of the material was then fractionated by preparative electrophoresis in Sephadex G-25 blocks.

Electrophoresis was carried out for 15–36 hrs. varying in the different cases. A representative result is given in Figure 2. From all preparations a protein rich and slowly migrating protein fraction was obtained and in addition a faster and complex peak of lower protein concentration. From each protein peak 2 pools of fractions were taken as illustrated by the Figure. The pooled materials were analysed as described below.

Serological, immuno- and physicochemical characterisation of the electrophoretic fractions.

Approximately 2/3 of each pool of fractions from the preparative electrophoresis was concentrated approx. 10-fold by ultrafiltration. This material, control IgG preparations and aliquots of the unfractionated digests were analysed by immuno-electrophoresis. The protein concentrations of the fractionated and concentrated material varied between 0.026 and 0.38 g/100 ml. The protein concentration of the fractions C and D were 2–13 times higher than that of the respective fractions A and B. When the digests, the pools A–D and the control IgG preparations were analysed with the anti IgG AO, EN and IN sera, the digests were seen to contain fast and slow components with electrophoretic mobility higher and lower than that of undigested IgG, respectively. In the case of the digest of IgG AO, absence of cross reaction between the fast and slow fragment was evident (Figure 3 a). Cross reaction or its absence was difficult to delineate for the components of the other

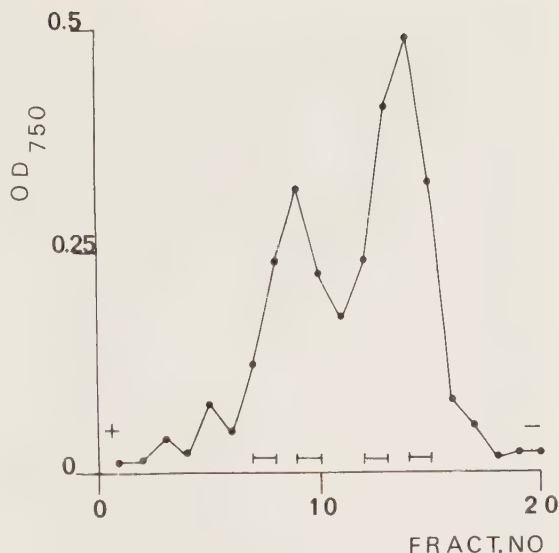


Fig. 2. Preparative electrophoresis in Sephadex G-25 block of gel filtered papain digest of IgG AO. The ordinate gives the optical density at 750 nm obtained at protein determination according to Lowry. The markings above the abscissa denote pooling of fractions (preparation A, extreme left—D, extreme right).

digests, since there was material present between the components with the mobility of undigested IgG. At least the fast components cross reacted with the IgG like material. When the pools A–D were examined with the same antisera (shown for AO in Figure 3 b and c) slow component was demonstrated in the pools C and D but not in A and B. In addition the fractions C and D were in each case seen to contain material precipitating near the antigen well. This material will provisionally be referred to as the “well-near” component. In the fraction C of AO the well near component predominated and only a faint precipitin line from the slow component was discernible. The pools A and B from AO and EN gave wavy precipitin lines, indicating presence of 2 subfractions. In mixtures of A and D materials absence of cross reaction between the fast and slow components was noted. The same applies to the fast and “well-near” components in the case of AO. In this cross reaction between the slow and “well-near” component was obvious. The

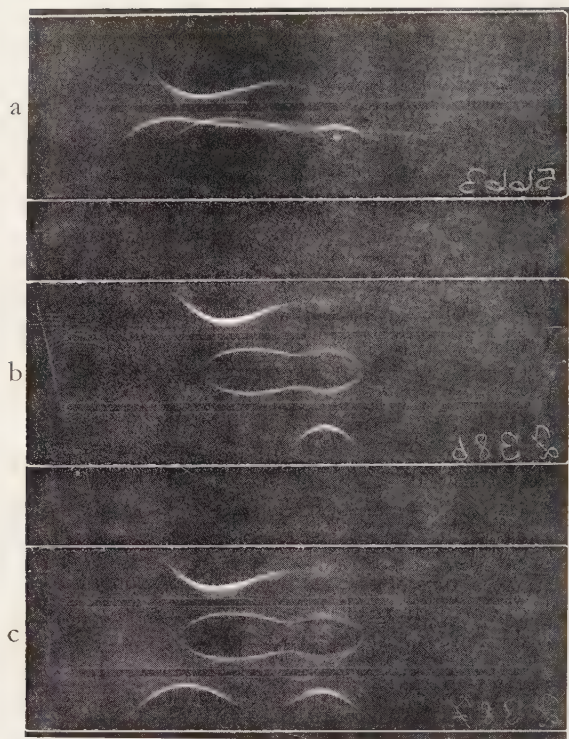


Fig. 3. Immunoelectrophoresis of control IgG AO preparation, papain digest of IgG AO and preparative electrophoresis fractions A—D from the gel filtered digest. Anti-IgG AO serum in the troughs. Slide a) control IgG (top), digest (below). Slide b) control IgG (above), fraction A (centre), fraction C (below). Slide c) control IgG (above), fraction B (centre), and fraction D (below). The lower precipitin line seen in slide b) represents the "well-near" component; it occurs in a similar position in slide c).

"well-near" component in the EN and IN material was too faint to allow conclusions regarding cross reactivity. Reaction against undigested IgG was discernible even at a protein concentration amounting to $1/10$ of that of AO D, $1/40$ of that of EN D and $1/35$ of that of IN D. The anti-IgG A and the anti- γ -chain sera precipitated the fast but not the slow or the "well-near" components. The anti-light chain serum gave precipitin lines against the slow components from EN and IN but not with certainty against the slow material in AO D. However, employing anti-light chain serum and with light chains in the slit opposite the antiserum basin, the slow AO component was seen to react. Reaction was not obvious between the anti-light chain serum and the "well-near" components. The anti-light chain serum gave no reaction against the fast components with either of the techniques.

As described below, the materials C and D were seen to contain antibody activity which was absent or weak in the other fractions.

In order to elucidate the nature of the active principle in the pools D, these were investigated in inhibition experiments with the antisera using the plate titration techniques. The antisera employed were: anti-IgG A and anti-light chain. Normal rabbit serum was used as a control and in addition transferrin plus anti-transferrin for AO and EN material and BSA plus anti-BSA for IN material, as unspecific precipitate controls. Other controls were: serial dilutions (saline) of the material to be tested, transferrin, BSA, the rabbit sera and saline. All preparations applied to the plates contained dextran sulphate and calcium chloride at the same concentrations. Equal amounts of D material and rabbit normal or antiserum or saline were mixed before application onto the plates. For AST determination $5 \mu\text{l}$ applications were used and $10 \mu\text{l}$ applications for ASTA assay. The D material was present in the mixtures at a protein concentration of $0.002 \text{ g}/100 \text{ ml}$ and the same concentration was chosen for transferrin and BSA. The results were: in all cases the anti-IgG A serum

gave a moderate decrease in haemolysis inhibition zone diametres whereas the anti-light chain serum inhibited almost completely. The unspecific precipitation controls were negative with the exception of AO material where some decrease in zone diameter was obvious. The decrease was, however, less than that given by the 2 other sera. Normal rabbit serum gave no depression in haemolysis inhibition. The other control preparations did not inhibit haemolysis.

The D materials were then gel filtered and the chromatographic fractions assayed for antibody activity with the plate methods (shown for AO in Figure 1). No antibody activity was demonstrated at the characteristic position of IgG in the chromatogram. Instead all antibody activity was localised in a peak, the R_{ribo} of which exactly coincided with

that of the main protein peak from the whole digests previously analysed in the same respective columns. At tube titration no antibody activity was observed in the region of IgG whereas activity in the peak region was confirmed.

Table 1 gives the result of titrations on the unconcentrated materials A—D together with determinations on the control IgG preparations. In each case the specific AST or ASTA activity of the C and D materials exceeded that of the A and B fractions. The first mentioned materials gave approximately the same value. These were for AO and IN approx. 80—100 per cent of the value for the control IgG, whereas the ratio was approx. 5 per cent for the EN fractions. In no case was activity demonstrable in the A fractions, in spite of subsequent concentration.

In another experiment the fractions A, or A plus B, D and mixtures of the fractions

Table 1. *Specific AST or ASTA activities (U/OD₂₈₀/ml) of fractions from preparative electrophoresis and of control IgG preparations.*

Material	Case	AO	EN	IN
IgG		1060	811	14
A		<8.7	<3.1	<0.83
B		48	<8.0	<0.53
C		1000	33	12
D		770	42	16

No antibody activity was discernible in the fractions AO, EN and IN A or in EN and IN B.

were titrated (Table 2). The A or A plus B fractions were present in 4- to 8-fold excess (w/w) over the D fractions in the mixtures. No activity was proved in the A or in the A plus B fractions and these materials in the mixtures did not inhibit the activity of the D fractions.

DISCUSSION

Gel filtration of the papain digests showed that almost all IgG had been cleaved by the enzyme. The rapid degradation of IgG AO is remarkable and contrasts to the relative stability towards enzymatic attack shown by the other AST active M-component. The susceptibility of IgG IN to papain action was intermediate compared to that of IgG AO and EN. The slow fragment, demonstrated in the preparative electrophoresis fractions C and D and virtually absent from the fractions A and B, was shown to consist of the Fab fragment as judged from its electrophoretic mobility, the absence of precipitin reaction with the anti-IgG A and the anti- γ -chain antisera (Cohen 1963) and its demonstrated content of light chains. The fast fragment, prevailing in the electrophoretic fractions A

Table 2. *AST or ASTA titre of fractions and mixtures of fractions from preparative electrophoresis*

Case	AO				EN		IN		
Material	A	D	A+D	A+B	D	A+B+D	A	D	A+D
Titre U/ml	<1	1.5	1.9	<1.3	1.2	1.2	<1.7	4.5	4.5

and B, was diagnosed as a Fc fragment by its electrophoretic mobility and its precipitin reaction with the anti-IgG A and the anti- γ -chain sera. As expected, cross reaction between the fast and slow components was not demonstrated. The "well-near" component was shown in the case of AO to cross react with the Fab fragment, and absence of cross reaction with the Fc fragment was obvious. The nature of the fragment is unclear, but it may possibly be an Fd fragment.

AST or ASTA activity was demonstrated in the C and D fraction; and the B fraction from AO but in neither case in the A fraction. The antibody activity in the D preparations could not be due to undigested IgG. This was shown by the gel filtration experiments, where activity was only demonstrated in material of lower molecular size than that of IgG. The immunoelectrophoretic findings were also incompatible with the presence of IgG in amounts sufficient to account for the observed antibody activities in the D preparations. The activity in the fraction AO C, which seemed to contain little Fab fragments suggested in this case that the "well-near" component contained AST activity. The marked suppression of haemolysis inhibition zone diameters obtained with the anti-light chain antiserum in all cases suggests that the Fab fragments were antibody active.

The A preparations, by immunoelectrophoretic criteria consisting of pure Fc fragments, showed no AST or ASTA activity. This also applies to the fraction EN B. Even when present in molar excess, the Fc fragments did not block the antibody activity from the D preparations.

The specific activity of the EN C and D materials was lower than that of the control IgG preparation. This means that all of the AST activity of the IgG molecule was not accounted for by the C and D material. The discrepancy may be misleading, however, since the AST activity only represents secondary manifestations of the reaction between lysin and the M-component, *i.e.* inhibition of lytic enzyme activity. Thus a comparatively low recovery of AST activity may be compatible with a quantitative recovery of active sites. This explanation is unlikely in the case of the Fc fragments, since, as mentioned, they were unable to block the action of the D material. The finding of antibody activity in the Fd half of the M-components implicates that these IgG molecules are at least bivalent.

SUMMARY

Preparations of Fc and Fab fragments were obtained by gel filtration and preparative electrophoresis of papain digests of IgG AO, EN and IN. The Fc fragments seemed pure while the Fab fragments were contaminated with unidentified material. In the case of AO this material was shown to be antigenically related to the Fab fragments but unrelated to the Fc fragments. With the techniques used, the Fc fragments proved antibody inactive, whereas activity was demonstrated in the Fab fragments. In the case of AO the unidentified material was probably antibody active.

IgG AO was highly susceptible to papain action, IgG EN was relatively resistant and IgG IN of intermediate susceptibility.

IV. Properties of the polypeptide chains of the M-components AO, EN and IN

As has been pointed out in the Introduction studies have earlier (Sjöquist and Zettervall 1966, Zettervall 1967 b) been carried out on the properties of the polypeptide chains of the three M-components. Thus the specific antibody activity of the heavy and light chain preparations was found to amount to approx. one per cent of that of the respective M-component preparations. On comparison the specific AST activity of the heavy chains of the M-component EN was found to probably exceed that of the light chains from the same source. On the contrary several experiments with the material AO seemed to indicate a slight excess of specific AST activity in the light chains over that of the heavy chains. No definite conclusion was reached as regards the ratio of specific activities of heavy and light chains from the M-component IN because of the low levels of antibody activity encountered in analysis of this material. The possibility could however not be ruled out that the antibody activity of the polypeptide chains was due to contamination with complementary chains or undissociated IgG. In recombination experiments with autologous heavy and light chains from the three M-components, potentiation of antibody activity was noted in each case. When heavy and light chains from the same IgG preparation were mixed and the dissociating condition (acid pH) removed, the specific antibody activity of the mixture was found to markedly exceed the specific activity value, calculated from that found in isolated preparations of the chains and the known concentration of these in the reconstituted mixture. Potentiation of antibody activity in conditions known to favour recombination of chains to four chain molecules is a typical finding for animal

antibodies (Franěk and Nezlin 1963, Edelman et al. 1963).

In homologous recombination experiments, where chains from AO and EN were used together with complementary chains from other IgG, comparatively little, or no potentiation was encountered. No definite advantage was obtained in crosses where both complementary chains were derived from AST active IgG, either poly- or monoclonal. The polyclonal chains used in these investigations (from IgG prepared from serum SE) gave minor or no potentiation in autologous recombination experiments. Hence, in recombination experiments, the M-component material seems to yield to the pattern considered as characteristic for antibodies, whereas the polyclonal human preparation SE does not. However, the relatively high specific antibody activity of the light chain preparation AO compared to that of the autologous heavy chains, may disturb the analogy with the animal antibodies. For these, heavy chains have usually been found to possess higher antibody activity than the complementary chains (Fleischman et. al 1963, Utsumi and Karush 1964, Porter and Weir 1966) although exceptions from this rule seem to exist (Goodman and Donch 1965).

So far, the antibody active material in reconstituted chain mixtures from the M-components AO, EN and IN have not been characterised in physico- and immunochemical terms. This was the object of the investigations described in the present Chapter, *i.e.* identification of the antibody active principle formed in such recombination experiments. Similar investigations are also given on the antibody active material in iso-

lated chain preparations from the M-components under study.

MATERIALS AND METHODS.

IgG from serum EN A was prepared by chromatography in DEAE-Sephadex A-50 with phosphate 0.0175 M, pH 6.50 as starting and eluting buffer. *IgG* GW was prepared by the same technique from a human G-myeloma serum. The preparation had a specific AST activity of <1 AST U/OD₂₈₀/ml. *IgG* from serum AO B was obtained by chromatography in DEAE-Cellulose (Serva) using the same buffer; 5–15 ml of the sera were fractionated. Column dimensions were 2 × 20 cm and flow rates 10–30 ml/hr. *IgG* from serum IN was the preparation *IgG* IN V, described in Chapter II. When the samples were analysed at a protein concentration of 1 g/100 ml in immunoelectrophoresis with polyvalent anti-human serum, only *IgG* could be demonstrated. During cellulose acetate electrophoresis employing the same protein concentration the preparations seemed to contain pure M-components. *IgG* Kabi was used as a source of normal polyclonal heavy and light chains.

Radio-iodine labelling of IgG was carried out according to Bocci's (1964) modification of Hunter and Greenwood's (1962) Chloramine T method. The Chloramine to protein ratio was 1 : 200 and reaction time 30 min. After addition of carrier iodide, free radioisotope was removed by gel filtration in Sephadex G-25 "Fine" (column 1 × 17 cm, 0.0175 M, pH 6.50 phosphate, flow rate appr. 15 ml/hr). The average extent of labelling was less than 1 iodine atom per molecule of *IgG*. The specific radioactivity of the labelled *IgG* preparations was between 10^5 and 10^7 counts per minute/unit absorbency at 280 nm (cpm/OD₂₈₀/ml). With the exception of *IgG* Kabi and *IgG* GW which were not iodinated, one aliquot of each *IgG* preparation was labelled with ¹³¹I and another at the same time with ¹²⁵I.

Preparation of heavy and light chains. Reduction

(tris-HCl 2 M, pH 8.0; 2-mercaptoethanol 0.5 M, 1 hr) and alkylation (iodo-acetic acid 0.55 M in same buffer, 20 min.) have been described elsewhere (Zettervall 1967 b). Part of the material was dialysed against 0.05 M tris-0.15 M NaCl, pH 7.2. The remainder was dialysed against 0.1 M formic acid in the cold for 18–30 hr after which heavy and light chains were separated by gel filtration in Sephadex G-200 40–120 μ in the same buffer (Sjöquist and Vaughan 1966). In the experiments with monoclonal *IgG* the column dimensions were 1.5 × 85 cm and the flow rates between 2 and 3 ml/hr. Chains from *IgG* Kabi were separated in a 4 × 80 cm column at a flow rate of approximately 20 ml/hr. Fractions were taken each hr. and read at 280 nm and appropriate pools of fractions corresponding to the respective heavy and light chain peaks were collected. For each *IgG* material ¹³¹I and ¹²⁵I labelled chains were prepared simultaneously in parallel columns.

Acrylamide gel electrophoresis was used for characterisation of heavy and light chain preparations. Chain preparations in 0.1 M formic acid or mixtures of chain preparations of an OD₂₈₀ of 0.4–0.7 were concentrated 5- to 10-fold by lyophilisation and subsequently dissolved in urea-SDS-phosphate sample buffer (Chapter I). Part of the preparation was saved for counting while other aliquots were electrophoresed under the conditions described in Chapter I. When radioactive material had been thus fractionated, 0.2 cm gel segments were cut, using a metal comb as a spacer. *IgG* preparations were lyophilised from 0.0175 M, pH 6.50 phosphate buffer and dissolved in $\frac{1}{2}$ –1 volumes of sample buffer before electrophoresis. In one experiment, *IgG* EN in the same phosphate buffer was mixed with 10 volumes of 0.1 M formic acid and kept overnight at room temperature before lyophilisation and electrophoresis at its original protein concentration.

Recombination of polypeptide chains. Heavy (H) and light (L) chains in 0.1 M formic acid were mixed at the mass ratio H/L of 4/3 as deter-

mined from measurements of the OD_{280} of the chain preparations. The same specific extinction coefficient, *i.e.* 13.6, was assumed for the 2 kinds of material. The optical densities ranged between 0.2 and 0.6. The mixtures, and preparations of the chains used in the mixtures, were simultaneously dialysed in the same bath for 36–50 hr at $+5^{\circ}\text{C}$. At least a hundredfold volume of 0.05 M tris-0.15 M sodium phosphate, pH 7.2 was used as outer solution; the dialysis buffer was changed once. Protein concentration was determined by spectrophotometry at 280 nm, using the last dialysis buffer portion as a blank. The AST or ASTA activity of the samples was determined and their specific antibody activity in AST or ASTA U/ OD_{280}/ml was calculated. In some instances, the dialysed chains were mixed and the preparations analysed simultaneously according to the same principles. Potentiation of antibody activity in chain mixtures was calculated from the formula,

(observed specific antibody activity of chain mixture)/(calculated specific activity of mixed chains).

The denominator is calculated from the sum of the antibody activities of the chains in the mixture, using the figures for the specific antibody activities of the isolated chain preparations and the known proportion of heavy and light chains in the mixture.

Gel filtration. Protein samples in neutral buffers, such as radio-iodinated IgG, reconstituted mixtures of heavy and light chains or isolated chain preparations in such buffers were analysed by gel filtration in Sephadex G-200, 40–120 μ , equilibrated with PBS. The riboflavin marker (Chapter I) was always included in the samples to be fractionated. Before application of the samples (0.5–1.0 ml) to the columns an aliquot of the material had been removed for counting; 3 columns of diameter 0.9–1.1, length 90 cm were run as described in Chapter I. In the autologous recombination experiments dialysed chain preparations and chain mixtures were chromatographed simultaneously. The same applies

to the 2 kinds of chain mixtures in the homologous experiments. In these, however, free chain preparations were usually not gel filtered.

The anti-light chain, anti-IgG A and anti-IgG AO antisera have been described in Chapter I. Anti-IgG AO was absorbed with an excess of polyclonal normal human light chains. The sera were not delipidised.

Serological methods. AST activity was usually titrated with the ordinary tube method or, in some instances, with the micro tube method. The choice of method is specified in each instance. ASTA activity was determined by the tube method. The plate methods were used for identification of antibody activity in chromatographic samples and in inhibition experiments with antisera. In the AST inhibition experiments equal volumes of antiserum and test solution were mixed; 5 μl of the mixture was then immediately applied onto the blood-agar diffusion plate.

Conventions. Chain preparations are referred to by self-explanatory abbreviations *viz.* H(AO), L(EN), etc. If needed the symbols include the iodine label, *e.g.* $H_{125\text{I}}$. Iodine labelled IgG preparations are similarly denoted. Chain mixtures are given as $H_{131\text{I}} + L_{125\text{I}}$, etc. Preparations of chains, mixed in formic acid and subsequently dialysed against tris-NaCl buffer will be referred to as chain mixtures or reconstituted chain mixtures. Preparations of chains, mixed after previous dialysis, are denoted as mixtures of chains.

RESULTS.

Preparation and analysis of heavy and light chains.

Separation of heavy and light chains. Fig. 1 is representative for the elution curves and pooling of fractions in the heavy and light chain separations. The variation noted in the shape of the elution curve in different experiments affects mainly the leading composite peak and probably reflects different degrees of heavy chain aggregation and/or presence of undissociated IgG. In collecting heavy chains,

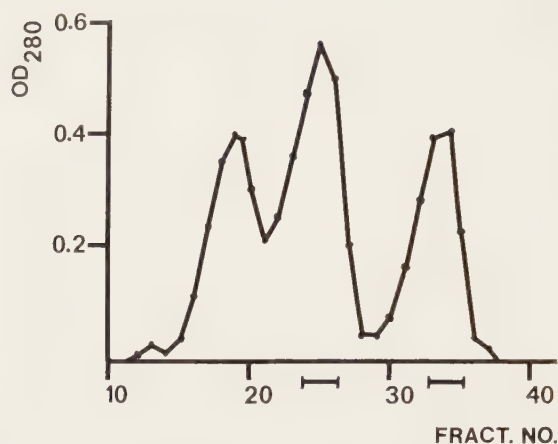


Fig. 1. Separation of polypeptide chains from reduced and alkylated IgG EN (20 mg) in Sephadex G-200-0.1 M formic acid. Column dimensions were 1.5×85 cm and fraction volumes 2.6 ml.

the ascending part of the leading peak was avoided.

Acrylamide gel electrophoresis of IgG and of chain preparations. Fig. 2 a—c gives representative counting patterns obtained, when iodine labelled IgG and chain preparations from the same protein were electrophoresed in the acrylamide-urea-SDS system. The samples, analysed in the same gel, were in this case: L_{131I} (EN) mixed with approx. 1/10 (w/w) H_{125I} (EN); 131I labelled IgG EN, and the

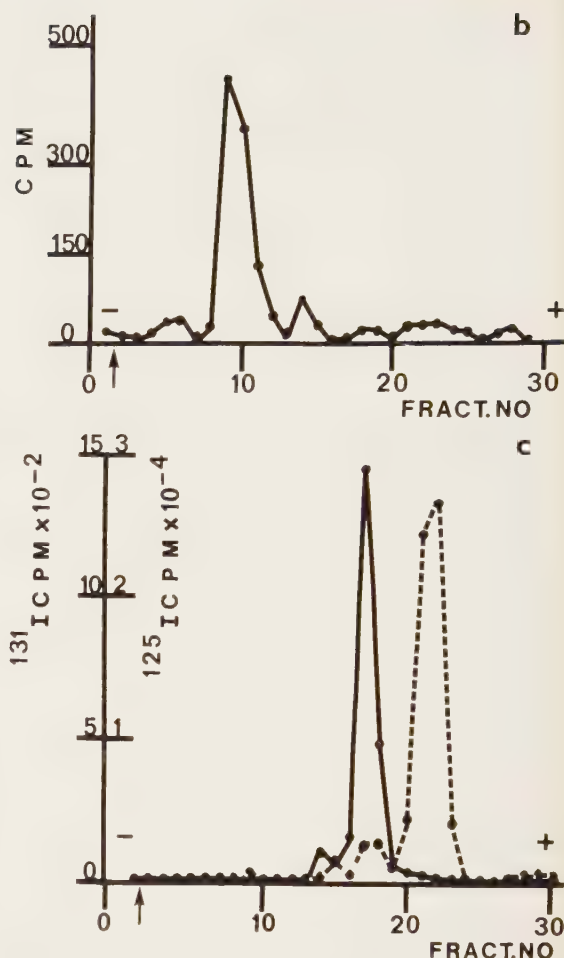
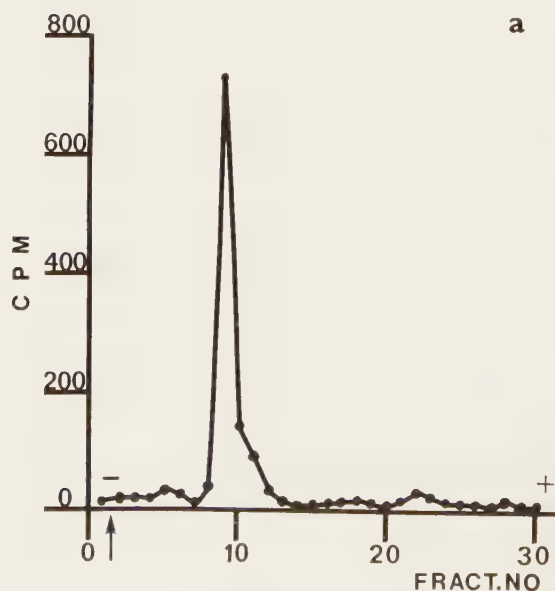


Fig. 2. Acrylamide gel electrophoresis of: a) IgG (EN) $_{131I}$; b) IgG (EN) $_{131I}$ treated with formic acid; c) a mixture of H_{125I} (EN) and L_{131I} (EN). The radioactivity of the gel fractions is given as counts per minute (cpm) on the ordinate. 131I radioactivity is denoted by the full line in a) and b) In c) the full line denotes 125I and the broken line 131I radioactivity.



same type of material, treated with 0.1 M formic acid as described in the Materials and methods section of this Chapter. As seen from the Figure the most anodic principal radioactivity peak from the L chain preparation was well separated from the main heavy chain peak. IgG gave a peak of mobility lower than that for heavy and light chains. Formic acid treatment did not grossly affect the mobility of the protein. The heavy chain preparation in addition showed one minor peak with lower electrophoretic mobility than that of the main fraction and the same applies to both

IgG preparations. The small peaks probably represent aggregated material. The IgG preparations gave shallow peaks in the heavy and light chain positions. The slow minor peak from L_{131I} corresponded in mobility to heavy chains, and was thus probably due to contamination with such material. If present a light chain peak from the heavy chain material was insignificant. No definite peaks from the chain preparations in the typical IgG position were discernible. Similar results were obtained with the other chain preparations. The chain preparations of IgG GW and pooled normal IgG were seen to contain insignificant amounts of contaminating complementary chains or IgG, as judged from the protein stain pattern (Fig. 3). When H (GW) and L (GW) were analysed at the same time with iodine labelled chains from IgG AO, or with unlabelled polyclonal chains, the electrophoretic mobilities of the respective chains from the different preparations correlated closely. However, on comparing the stain patterns the heavy and light chain stain from the normal IgG preparation seemed more diffuse than those from IgG (GW).

The total recovery of radioactivity in the gel fractions amounted to between 80 and 95 per cent of the activity applied to the plate. Calculation of the contamination with complementary chains was facilitated by the addition of the samples with chain preparations, labelled with the alternative iodine isotope. This is illustrated by the following example, which shows the method of calculating the degree of contamination of L_{125I} chains with H_{125I} ; H_{131I} is assumed to have been added to the preparation. The specific radioactivity (cpm/unit mass) is first assumed to be equal for both H_{125I} and L_{125I} . If the H_{131I} peak contains p_{131I} per cent of the total 131I label applied to the plate and p_{125I} per cent of the other label is found in the heavy chain position the mass ratio H_{125I}/L_{125I} in the sample is calculated as p_{125I}/p_{131I} . In this calculation the chain preparations have been assumed as

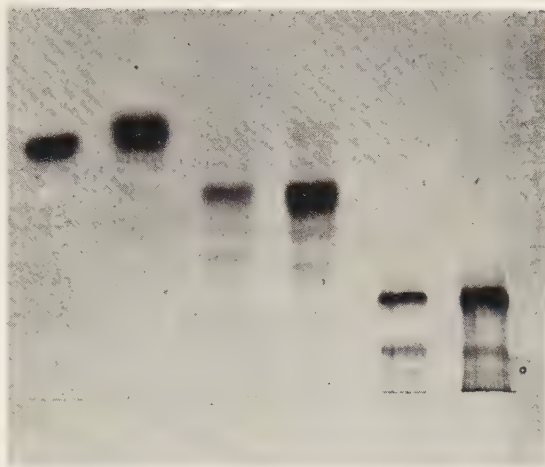


Fig. 3. Electrophoresis in acrylamide gel of polypeptide chains from IgG GW and pooled normal IgG. From left to right: L (GW), normal L chains, H (GW), normal H chains, IgG (GW) and normal IgG. The anode is at the top. The protein content of the samples was 0.15 mg.

nearly pure. With the specific radioactivity of H_{125I} and L_{125I} denoted as s_H and s_L , respectively and now disregarding the restriction $s_H/s_L = 1$, the percentage of contaminating H_{125I} chains in the L_{125I} preparation is $(p_{125I}/p_{131I}) \times (s_L/s_H) \times 100$. The contamination with IgG was calculated from the radioactivity recovered from the position of IgG. This position as well as the percentage of recovery of the total IgG radioactivity in the gamma globulin peak was obtained at parallel analysis of IgG in the same plate.

The contaminations of the chain preparations are given in Table 1.

Characterisation of IgG and of dialysed chains and autologous chain mixtures.

The IgG_{125I} preparations were characterised by gel filtration.

IgG, iodine labelled IgG and reduced, alkylated but not dissociated labelled IgG were titrated simultaneously, and their specific antibody activities calculated from optical density readings at 280 nm. The IgG preparations AO and EN were titrated both with the ordinary and the micro tube techniques.

Table 1. Contamination of chain preparations with IgG and complementary chains.

Case	Chain preparation	Contaminating material (per cent w/w)		
		H-chains	L-chains	IgG
AO	H125 _I		14	0.7
	L125 _I	1.1		0.04
	H131 _I		13	0.9
	L131 _I	1.5		0.07
EN	H125 _I		2.3	0.2
	L125 _I	0.9		0.05
	H131 _I		2.8	1.1
	L131 _I	12		1.3
IN	H125 _I			0.1
	L125 _I	10		0.02
	H131 _I			0.8
	L131 _I	1.3		2.2

Chain preparations and chain mixtures were analysed as described in Materials and methods. In the case of AO and IN, mixtures of dialysed chains were similarly investigated, but only the IN material was gel filtered.

Gel filtration of iodine labelled IgG AO (Fig. 4) EN and IN. The radioactivity peaks emerged at a R_{ribo} from 0.550 to 0.575

Presence of aggregated IgG was evident from the shape of the radioactivity curves at the leading part of the main peaks. Aggregated radioactive material seemed to constitute only a minor part of the total iodine labelled protein recovered from the columns. The recovery of radioactivity in the fractions was 60 to 100 per cent of that applied to the columns. This contrasted with the recovery of heavy and light chain radioactivity in the subsequent experiments where the figures were much lower, i.e. less than 50 per cent.

Case AO.

Specific AST activity of IgG and of reduced and alkylated iodinated IgG. (Table 2, Experiment 1). Without iodination IgG had a specific

AST activity by the ordinary tube method nearly coinciding with that of IgG AO II in Chapter II. With the micro modification approx. half the value was obtained. Iodine labelling did not adversely affect the AST activity; on the contrary, compared with untreated IgG the figures for the iodinated proteins were slightly higher using the ordinary titration method. However, this difference is not considered definite. As for untreated IgG, the titres obtained with the micro method were lower than those found at ordinary titrations. Reduction and alkylation decreased the specific AST activities to about 35 per cent of the figure for iodinated IgG, when the results found with the ordinary titration method are considered. There was no real difference between the specific AST activities obtained

Table 2. Case AO. Specific AST activity (U/OD280/ml) of IgG, polypeptide chains, reconstituted chain mixtures and mixture of dialysed chains.

Exp.	Material	Specific AST activity	Potentiation	% reconstituted of Ab activity	
1	IgG	1760	909		
	IgG125 _I	1865	835		
	IgG125 _I , r+a	704	833		
	IgG131 _I	2133	1342		
	IgG131 _I , r+a	743	624		
2	H125 _I		4.52		
	L125 _I		27.7		
	H131 _I		11.8		
	L131 _I		15.5		
	H125 _I +L131 _I	113	12.3	18.1	
	H131 _I +L125 _I	297	16.0	47.6	
	* H131 _I +L125 _I		20.2	1.0	
3	H125 _I		7.64		
	L125 _I		13.5		
	* H125 _I +L125 _I		32.1	3.0	

Figures in italics denote results from micro tube titrations; r+a= reduced and alkylated.

* mixture of dialysed chains.

with either titration method, in contrast to that found for untreated or iodine labelled IgG. *Specific AST activity of heavy and light chains, chain mixtures and mixtures of chains* (Table 2, Experiment 2,3). In Experiment 2 chains and reconstituted chain mixtures from the 2 iodinated IgG preparations were dialysed together. The samples were titrated together with a mixture (H/L=1/1, w/w) of aliquots from the dialysed chain preparations H_{131I} and L_{125I} . The specific AST activity of H_{125I} was much lower than that of H_{131I} . The reverse is true for the respective L chain preparations. For both sets of complementary chains the specific AST activity of the light chains exceeded that of the heavy chains. The specific activity of any chain preparation was less than 4 per cent of that of reduced and alkylated IgG. For both reconstituted mixtures, *i.e.* $H_{125I} + L_{131I}$ and $H_{131I} + L_{125I}$, the specific AST activity figure was several times higher than the values found for the isolated chain preparations, which is illustrated by the potentiation factors, 12.3 and 16.0. In the combination $H_{131I} + L_{125I}$ this activity nearly amounts to 50 per cent assuming that all heavy chains in the mixture recombine with light chains to four-chain molecules of specific activity equal to that of reduced, alkylated and undissociated IgG. In the case where the chains were mixed after

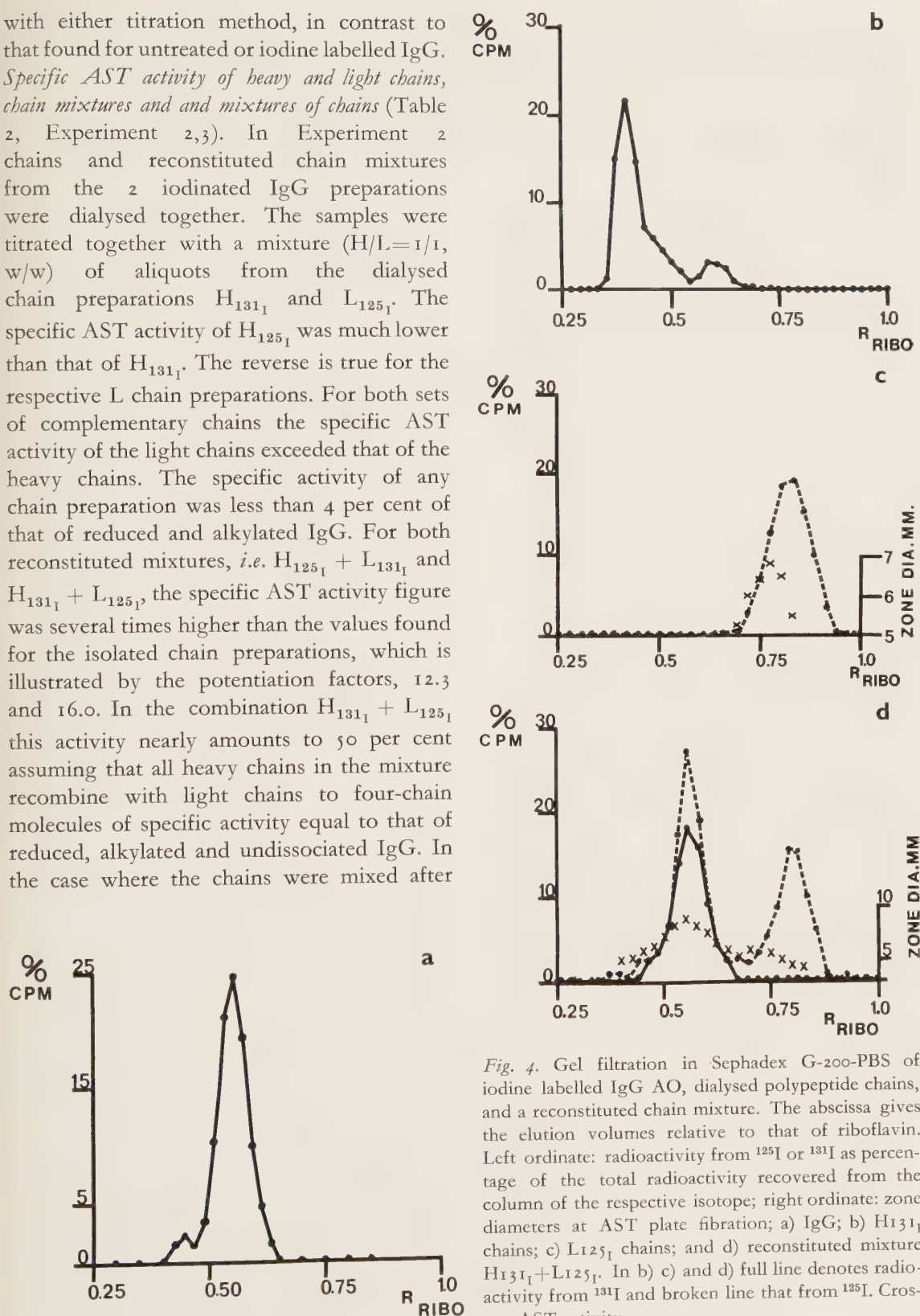


Fig. 4. Gel filtration in Sephadex G-200-PBS of iodine labelled IgG AO, dialysed polypeptide chains, and a reconstituted chain mixture. The abscissa gives the elution volumes relative to that of riboflavin. Left ordinate: radioactivity from ^{125}I or ^{131}I as percentage of the total radioactivity recovered from the column of the respective isotope; right ordinate: zone diameters at AST plate fibration; a) IgG; b) H_{131I} chains; c) L_{125I} chains; and d) reconstituted mixture $H_{131I} + L_{125I}$. In b) c) and d) full line denotes radioactivity from ^{131}I and broken line that from ^{125}I . Crosses: AST activity.

previous dialysis no enhancement of AST activity was found as seen for the combination, $H_{131I} + L_{125I}$. However, in Experiment 3, where dialysed chains were mixed in the proportion (w/w) $H/L = 0.88$ potentiation was found.

Characterisation of dialysed chains and chain mixture by gel filtration (Fig. 4). The dialysed chain preparations H_{131I} , L_{125I} and the chain mixture, dialysed after mixing of the chains in formic acid, were analysed by gel filtration. These preparations are the same as those given in Table 2, Experiment 2.

The heavy chain preparation H_{131I} , gave a main radioactivity peak with a R_{ribo} of 0.395 followed by a minor peak with R_{ribo} of 0.595. Both peaks appear complex. Plate titration showed a questionable activity in a few fractions preceeding the principal peak and a weak, but definite activity in the region R_{ribo} 0.375–0.585, with a maximum at 0.457. Titration with the micro tube method showed one activity peak at $R_{\text{ribo}} = 0.30$.

Since the radioactivity of this fraction, *i.e.* the one which was immediately followed by the first definitely radioactive fraction of the main peak, was not significantly higher than the background, no specific AST activity could be calculated. Hence the heavy chain preparation seemed to contain 2 AST active fractions; one poorly diffusible but more antibody active than the other, more diffusible fraction.

The light chain preparation L_{125I} , gave one slightly skewed count peak with maximum at $R_{\text{ribo}} = 0.834$. AST activity was clearly demonstrable in the region 0.611–0.863 with a maximum at 0.777. This is near the characteristic position of the AST activity peak (0.715–0.740) given by Fab fragments (*cf.* Chapter III). These findings were confirmed at tube titration. A specific AST activity was calculated using the titre found for the most AST active fraction and the specific radioactivities of the L-chains. The figure was 31

AST U/OD₂₈₀/ml. In order to further characterise the AST active material, an aliquot of the same dialysed L_{125I} preparation that had been gel filtered was investigated in inhibition experiments with the rabbit sera, anti-IgG AO, anti-light chain, anti-IgG A, and normal rabbit serum. The 3 first-mentioned sera gave inhibition of the AST activity of the preparation as seen from the decrease of the haemolysis inhibition zone diameters, while the normal serum did not. The first antiserum was the most potent and gave virtually complete extinction; the anti-IgG A serum was the least active. When the dialysed preparation L_{131I} was similarly tried against anti-IgG AO and anti-light chain sera, the findings were similar. When gel filtered, an AST maximum was found at R_{ribo} 0.740; the radioactivity peak

had its highest value corresponding to 0.783, *i.e.* later in the chromatogram than the peak antibody activity.

The reconstituted mixture, $H_{131I} + L_{125I}$. The Figure shows that L-chains and most of the H-chains have been transferred from their characteristic positions in the chromatograms of free dialysed chains. The heavy and light chain labels both gave a peak with a maximum at $R_{\text{ribo}} = 0.558$. Shouldering of heavy chain label in front of the main peak is apparent. The majority of the light chains recovered from the column appeared together with the H chains in the peak while the remainder occurred at the free light chain position. The calculated molar ratio H/L in the peak fraction was 1.0 and the same figure was obtained from the total radioactivity of the peak. It thus appeared as if the material in the fractions from the peak was homogenous in its composition of heavy and light chains.

Titration with both the plate and the micro-tube techniques showed AST activity in the fractions from the peak, following the count profile. The protein concentration of the peak fraction and of the pooled material from the peak was calculated from the sum of the radioactivity counts of the heavy and

light chains, using the specific radioactivities of the isolated chain preparations. The specific AST activities, obtained from these figures in conjunction with the titre values were 508 for the peak fraction and for the pooled material 501 AST U/OD₂₈₀/ml.

Case EN.

Specific AST activity of IgG, and of reduced, alkylated and iodinated IgG. (Table 3, Experiment 1).

As was the case for untreated IgG AO, the corresponding preparation gave a substantially lower specific AST activity in the micro-tube titration system than in the case of the ordinary titration technique. A considerable decrease in titre was displayed by both iodinated IgG preparations. The micro method also gave lower figures in this case, about 50 per cent of those obtained by the other titration technique. Reduction and alkylation further decreased the specific antibody activity. As shown for IgG_{125_I} the values obtained with both methods agree. This probably also applies to IgG_{131_I}, since almost 50 per cent haemolysis was obtained in the first titration tube.

Specific AST activities of heavy and light chains, and chain mixtures (Table 3, Experiment 2). Definite titres were obtained for the heavy chain preparations; the specific AST activities agreed acceptably for the 2 preparations and were less than 1 per cent of the value for reduced and alkylated IgG. As no sign of activity was obtained for either of the light chain preparations and considering that the upper limits for specific activities thus found were approximately the same as the definite figures for the heavy chains, the specific AST activity of the light chain preparations was probably lower than that of the heavy chains. At least a tenfold potentiation of AST activity over that of free chain preparations was noted for both reconstituted chain mixtures, H_{125_I} + L_{131_I} and H_{131_I} + L_{125_I}. Recovery of antibody activity was comparatively low, approximately 9–13 per cent.

Table 3. Case EN. Specific AST activity (U/OD₂₈₀/ml) of IgG, polypeptide chains and reconstituted chain mixtures.

Exp.	Material	Specific AST activity	Potentiation	% recovered of Ab activity
1	IgG	1300	526	
	IgG _{125_I}	572	278	
	IgG _{125_I} r+a	295	287	
	IgG _{131_I}	624	304	
	IgG _{131_I} r+a	324	<333	
2	H _{125_I}	1.92		
	L _{125_I}	<2.53		
	H _{131_I}	2.25		
	L _{131_I}	<2.14		
	H _{125_I} + L _{131_I}	35.0	>17.4	13.2
	H _{131_I} + L _{125_I}	23.8	>10.0	9.0

Figures in italics denote results of micro tube titrations, r+a= reduced and alkylated.

Characterisation of dialysed chains and chain mixture by gel filtration. The same preparations as shown in Table 3, Experiment 2 were used in the gel filtration experiments.

The heavy chain preparation, H_{125_I}. The label appeared as a composite fraction with its maximum at R_{ribo} 0.450. No AST activity was found in the fractions.

The light chain preparation, L_{131_I}. The radioactivity curve appeared as a slightly skewed peak with maximum at R_{ribo} 0.825. The fractions were devoid of demonstrable AST activity.

The reconstituted mixture, H_{125_I} + L_{131_I}. Most of the radioactivity recovered from the column of the heavy chain label occurred in a peak at R_{ribo} 0.562. A minor part of the recovered L label was found in a peak with a maximum at exactly the same position. The main L chain label peak had its maximum at R_{ribo} 0.791. For a pool of fractions from the leading count peak the molar ratio between H and L chains was

calculated as 1.9. For the peak fraction itself the ratio was 2.0. AST titration with the plate method demonstrated antibody activity within the leading radioactivity peak, the maximum haemolysis inhibition coinciding with the peak of radioactivity. Ordinary tube titration of pooled material from the peak gave no definite AST titre, but a weak inhibitory effect was discernible in the first titration tube. The peak fraction gave, however, a definite AST titre. The specific AST activity of the peak fraction, calculated from its AST titre, heavy and light chain radioactivity and specific radioactivities of the chains, was 148 AST U/OD₂₈₀/ml.

Case IN.

Specific ASTA activities of IgG and of reduced and alkylated, iodinated IgG (Table 4, Experiment 1).

The values obtained for untreated and radioiodinated IgG were almost identical. Reduction and alkylation resulted in a minor depression of specific ASTA activity.

Specific ASTA activity of heavy and light chains, chain mixtures and mixtures of chains (Table 4, Experiment 2 and 3).

No sign of ASTA activity was apparent in the light chains. A definite value was obtained for H_{125I} amounting to approx. 1.5 per cent of the value for reduced and alkylated IgG. The reconstituted chain mixture H_{125I} + L_{131I} was active and showed potentiation. Potentiation was encountered for the mixture of the same chain preparations (H/L = 1/1, w/w) dialysed before mixing. A similar result was obtained (Experiment 3) with the H/L ratio 1.33 (w/w).

Characterisation of dialysed chains, chain mixture and mixture of chains by gel filtration. The same preparations as given in Table 3, Experiment 2 were analysed with the exception of the mixture of dialysed chains H_{125I} + L_{131I} which was that of Experiment 3.

The heavy chain preparation, H_{125I}. The radioactivity appeared as a principal peak at R_{ribo} = 0.288 followed by trailing and a minor complex peak at approximately 0.50.

The light chain preparation, L_{131I}, gave a radioactivity peak at R_{ribo} = 0.745. Because of the

virtual absence of ASTA activity in the heavy and light chain sample, scanning of the fractions for ASTA content was omitted.

The reconstituted mixture H_{125I} + L_{131I}. The heavy chain label was found in a double peak, the leading part of which (R_{ribo} = 0.386) was

followed by a main peak with maximum at 0.545. The light chain label appeared in the same positions, but most of the light chain label recovered, appeared in a peak of R_{ribo} = 0.773. The calculated molar ratio of heavy to light chains in the fractions around R_{ribo} = 0.545 was 0.90 and in the peak fraction it was 0.84.

ASTA activity was found with plate titration coinciding with the principal heavy and

Table 4. Case IN. Specific ASTA activity (U/OD₂₈₀/ml) of IgG, polypeptide chains, reconstituted chain mixtures and mixture of dialysed chains.

Exp.	Material	Specific ASTA activity	Potentiation	% reactivity of Ab activity
1	IgG	29.3		
	IgG _{125I}	27.3		
	IgG _{125I} , r+a	19.6		
	IgG _{131I}	31.3		
	IgG _{131I} , r+a	24.5		
2	H _{125I}	<0.97		
	L _{131I}	<1.15		
	H _{125I} + L _{131I}	4.11	>3.92	21.7
	* H _{125I} + L _{131I}	1.40	>1.32	
3	H _{125I}	0.42		
	L _{131I}	<0.43		
	* H _{125I} + L _{131I}	2.0	>4.71	

* mixture of dialysed chains.
r+a = reduced and alkylated.

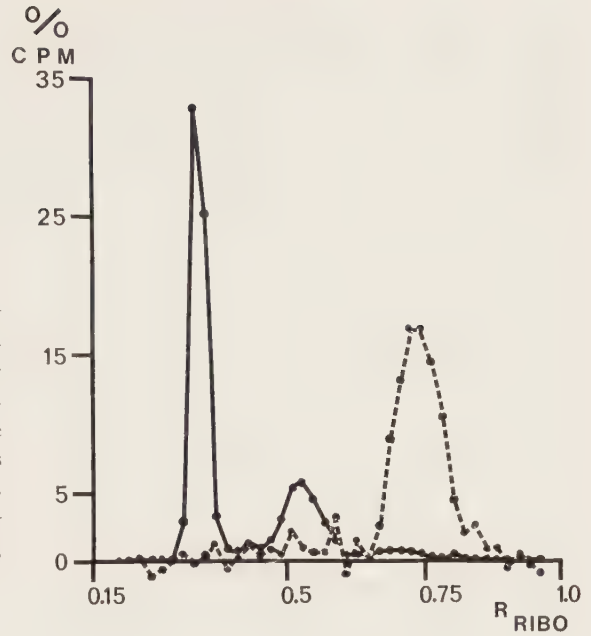


Fig. 5 Text: Gel filtration in Sephadex G-200-phosphate buffered saline of a mixture of H_{125}I (IN) and L_{131}I (IN). The chains were dialysed against tris-NaCl buffer before mixing. The abscissa gives the elution volume relative to that of riboflavin (R_{ribo}). The ordinate denotes the radioactivity from ^{125}I or ^{131}I as percentage of the total radioactivity recovered from the column of the respective isotope. Full line signifies radioactivity from ^{125}I and broken line that from ^{131}I . When free light chains (L_{131}I) were gel filtered radioactivity was not obvious in the fractions of $\text{R}_{\text{ribo}} < 0.6$.

Table 5. Specific AST or ASTA activities of chains and of chain mixtures at homologous recombination experiments.

Material	AST U/OD ₂₈₀ /ml	Potentiation	ASTA U/OD ₂₈₀ /ml	Potentiation
H_{125}I (AO)	7.64			
L_{125}I (AO)	13.5			
H_{125}I (AO) + L_{125}I (AO)	65.7	6.47		
H_{131}I (IN)			<0.72	
L_{131}I (IN)			<0.69	
H_{131}I (IN) + L_{131}I (IN)			4.37	>6.18
H_{131}I (IN) + L_{125}I (AO)	6.64	<1.15	<0.85	
H_{125}I (AO) + L_{131}I (IN)	3.73	<0.85	<0.75	
H_{131}I (AO)	11.8			
L_{131}I (AO)	37.6			
H_{131}I (AO) + L_{131}I (AO)	180	7.87		
H_{125}I (EN)	< 4.18			
L_{125}I (EN)	< 6.62			
H_{125}I (EN) + L_{125}I (EN)	22.6	>4.33		
H_{125}I (EN) + L_{131}I (AO)	36.2	2.25 > ; 1.96 <		
H_{131}I (AO) + L_{125}I (EN)	15.3	2.27 > ; 1.59 <		

In each of the 2 experiments all preparations were treated in parallel. AST titration was carried out with the micro tube method. When definite titre values were not obtained for chain preparations limits were set for the corresponding potentiation values using the upper specific antibody activity limit for the chains, or zero, respectively. The ASTA activity of the preparations AO and EN and the AST activity for the IN samples were assumed to be zero.

light chain peak. The maximum ASTA value corresponded to the fraction with highest heavy and light chain radioactivity. No antibody activity was evident inside the leading minor peak. The findings were confirmed by tube titration using a reduced amount of staphylolysin in the assay system, the absolute titre figures are therefore uncertain.

The mixture of dialysed chains ($H_{125_I} + L_{131_I}$).

The heavy chain associated radioactivity emerged in a leading major peak at $R_{\text{ribo}} = 0.333$ containing most of the eluted ^{125}I activity followed by a smaller component with its maximum at 0.529. A small amount of light chain label was observed in the region of this heavy chain peak (Fig. 4).

Characterisation of homologous chain mixtures

Homologous chain mixtures in formic acid were prepared with material from IgG AO, EN and GW. Similarly crosses were made between chains from IgG AO and IN. For comparison, autologous chain mixtures of the materials AO, EN and IN were included in the respective experiments. The dialysed chain preparations and chain mixtures were analysed according to the same principles as used in connection with the autologous recombination experiments. Free chain preparations and autologous chain mixtures were, however, not gel filtered.

Specific antibody activity of heavy and light chains and chain mixtures (Table 5).

Compared to what had been found for the autologous reconstituted mixtures the heterologous crosses were inefficient as regards potentiation of antibody activity. The most successful combinations were $H_{131_I}(\text{AO}) + L_{125_I}(\text{EN})$ and $H_{125_I}(\text{EN}) + L_{131_I}(\text{AO})$ (potentiation <2.27 , >1.59). In the crosses between chains from IgG AO and IN neither AST or ASTA activity was significantly enhanced, and the same applied to mixtures of the dialysed chains, not given in the Table. The crosses between AO and EN chains on one hand and GW chains on the other gave no poten-

tiation (chain mixtures). $H(\text{GW}) + L(\text{EN})$ showed no AST activity; since the chains were without demonstrable AST activity,, potentiation could not be calculated.

Characterisation of chain mixtures by gel filtration.

$H_{125_I}(\text{EN}) + L_{131_I}(\text{AO})$ and $H_{131_I}(\text{AO}) + L_{125_I}(\text{EN})$. Both preparations gave peaks containing both labels. R_{ribo} was 0.543 and

0.538, respectively. The rest of the light chain radioactivity was recovered in peaks of R_{ribo} 0.805 and 0.795. For both crosses, the

compound heavy and light chain peaks showed AST activity at plate titration. In addition, antibody activity was obvious in the free light chain peak from the combination $H_{125_I}(\text{EN}) + L_{125_I}(\text{AO})$ at R_{ribo} 0.740. At micro tube

titration definite titre values were not obtained. The localisation of AST activity was, however, substantiated by the partial streptolysin inactivation discernible. The specific AST activity of the main peak fractions from the 2 chromatographies was calculated as $<63 \text{ AST U/OD}_{280/\text{ml}}$.

$H_{125_I}(\text{AO}) + L_{131_I}(\text{IN})$ and $H_{131_I}(\text{IN}) + L_{125_I}(\text{AO})$. Major composite heavy and light chain radioactivity peaks were obtained with both preparations. The respective values of R_{ribo} was 0.526 and 0.520. AST or ASTA

activity was not found in the chromatographic fractions from either of the 2 heterologous mixtures.

$H_{125_I}(\text{AO}) + L(\text{GW})$ and $H(\text{GW}) + L_{125_I}(\text{AO})$. The heavy chain radioactivity from the first cross was apparent as a heterogenous peak with maximum at $R_{\text{ribo}} = 0.574$.

Insignificant radioactivity was observed at the position earlier found for free AO heavy chains, *i.e.* R_{ribo} approx. 0.400. $L_{125_I}(\text{AO})$

from the second cross appeared as a radioactivity peak with maximum at 0.800. No radioactivity was obvious in the fractions of $R_{\text{ribo}} < 0.65$. AST activity was not demonstrated in the chromatographic fractions.

H_{131I} (EN) + L (GW) and H (GW) + L_{131I} (EN). The findings were similar to those obtained in the crosses just described. The heavy chain associated radioactivity was found in a peak with its maximum at R_{ribo} 0.535. The light chain label from the second chain mixture occurred as a peak with a R_{ribo} of 0.828. No radioactivity was found at R_{ribo} < 0.65. Because of the low AST activities of the reconstituted mixtures, titration of the chromatographic fractions was omitted.

DISCUSSION

Antibody activity was demonstrated in heavy chain preparations from each of the M-components AO, EN and IN and in addition in the light chain samples from IgG AO. The question whether the activity was a genuine property of the respective chains or whether it was due to contaminations with other molecular species was not completely resolved.

In the case of L (AO), the gel filtration experiments conclusively show that at least most of the AST activity must be caused by contaminating material. There is still the possibility, however, that the light chains may possess the crucial antibody property, *i.e.* binding of antigen, in spite of absence of haemolysis inhibition. The molecular size of the AST active molecule as judged from its position in the chromatogram nearly coincides with that of Fab fragments (Chapter III). Also, the inhibition pattern with anti-IgG and anti-light chain sera was similar to that obtained with the Fab preparations, *i.e.* marked inhibition with the anti-light chain serum and less with the anti-IgG serum. It is conceivable that enzymatic splitting of IgG AO may have occurred during storage of the serum. For the heavy chains from the case AO, the gel filtration disclosed antibody activity in material greater in molecular size than IgG. The figure found for contamination with light chains is high enough to explain the activity as one of

reconstituted heavy and light chains. If such contamination is the explanation, the reconstituted molecules must be of higher molecular size than that of IgG to fit with the gel filtration data. Another possible explanation according to the electrophoretic findings is contamination with IgG. This is, however, unlikely since no antibody activity corresponding to the molecular size of IgG was obvious. Principally inhibition experiments with antisera may give a clue to the nature of the antibody active material in the heavy chain preparations. Practically this was not possible because of the poor diffusibility of the active principle, which constituted further evidence for its high molecular nature. Also in the case of heavy chains from case EN, contamination with IgG or light chains may be taken as an explanation of the observed antibody activities. For heavy chains from IgG IN, *i.e.* H_{125I} contamination with IgG as the sole explanation for the ASTA activity in the preparation is impossible according to the electrophoretic data. This is the more so since the values obtained at electrophoresis are probably overestimated due to inclusion of radioactivity from trailing material in the calculations. The figures found for light chain contamination in H(IN) were too uncertain to be taken into consideration. This was because the specific radioactivities of the light chain preparations proved to be very low, giving statistically uncertain count figures together with a high multiplicative correction factor for specific radioactivity in the calculations.

The autologous reconstitution experiments demonstrated in each case that heavy and light chains had recombined to aggregates of a molecular size equal to, or near that of native IgG. Further it was shown that reconstituted molecules were antibody active. In the cases where the specific activity of the reconstituted molecules could be calculated (AO and EN) the values were lower than those for the respective reduced, alkylated but not dissociated iodinated IgG preparations. The propor-

excess of unspecific protein present. The reasonably good agreement obtained with both types of titration methods in the case of reduced and alkylated material may be due to a charge effect, introduced by the alkylating agent, iodo-acetic acid.

SUMMARY

AST activity was found in the heavy chain preparations from IgG AO and EN and in the light chain preparations from IgG AO. ASTA activity was demonstrated in a heavy chain preparation from IgG IN. Upper limits for contamination of the chain materials with IgG or complementary chains were obtained at electrophoresis in acrylamide gels. Contamination as a cause of the observed antibody activities in the heavy chain preparations could not be ruled on. The AST activity in the light chain preparations from IgG AO was due to contamination with material resembling Fab fragments in its gel filtration characteristics and in its reaction with antisera. Formation of molecules containing heavy and light

chains was demonstrated in recombination experiments. In the autologous recombinations of chains from the antibody active M-components AO, EN and IN the heavy and light chain aggregates were antibody active. The same was the case with both hybrids between chains from the AST active M-components AO and EN. The similar crosses between chains from IgG AO and the ASTA active M-component IN were without demonstrable AST or ASTA activity. Definite potentiation of antibody activity was absent in these homologous crosses as well as in the homologous recombinations of chains from the AST inactive M-component GW with chains from IgG AO or EN. Mixtures of chains from IgG IN, dialysed against neutral buffer before mixing and the same type of mixture of chains from IgG AO showed potentiation of antibody activity. This may be due to formation of heavy and light chain hybrids.

The volume of the titration system was seen to be critical in the determination of AST activity of preparations of the M-components AO and EN but not critical at titration of the reduced and alkylated proteins.

V. Some properties of the M-component-haemolysin reaction

The work presented in this Chapter concerns some aspects of the anti-haemolysin activity of the M-components AO, EN and IN. Attempts are made to elucidate whether this activity is due to a reaction between the M-components and haemolysin and if so, whether this implies a permanent binding between the reactants as is characteristic for ordinary antibodies. In order to further correlate the properties of ordinary antibodies with those of the M-components comparison is made with the reactions obtained with polyclonal AST or ASTA material.

MATERIALS AND METHODS

Sera: The sera used were already described, SE in Chapter I, AO and EN B in Chapter II.

Protein preparations: Pooled human anti- α -staphylolysin IgG (IgG ASTA) and pooled normal human IgG were described in Chapter I and the AST and ASTA inactive G-myeloma globulin GW in Chapter IV. The IgG preparations AO, EN and IN were those given in Chapter IV.

Iodine (^{125}I) labelling of IgG GW was carried out with the technique described in Chapter IV.

Reduction and alkylation of the iodine-labelled monoclonal IgG preparations: the preparations IgG AO, EN and IN were the same as those used in Chapter IV and the procedure with IgG GW was that given in the same context. Low molecular reactants had been removed by dialysis against at least a 100-fold volume of either tris-NaCl buffer, or PBS.

Radio-iodinated human serum albumin (RIHSA) was described in Chapter I. The label was ^{131}I .

Kinetics of streptolysin- and α -staphylolysin inactivation by AST and ASTA.

AST: Serum SE and AO and the pooled normal human IgG preparation were diluted with PBS to an AST activity of 2 U/ml; serum EN was diluted to 4 U/ml. Then 0.5 ml aliquots of the preparations were added to 0.5 ml of PBS and the mixtures were incubated at 37°C for approximately 15 min. in order to achieve temperature equilibration. An amount (0.5 ml) of reduced (1/25 vol. 10 per cent sodium pyrosulphite, 30 min., room temperature) streptolysin, diluted to 1 combining U/ml was added to the tubes. These were further incubated for various lengths of time, after which 0.5 ml of 5 per cent sheep red cells in PBS were added. After 1 hr at 37°C, 2 ml of PBS was added, the tubes centrifuged and haemolysis assayed by spectrophotometry at 525 nm. A control tube, containing the same dilution of streptolysin (0.5 ml) and PBS (1.0 ml) was incubated in parallel. Of this 0.2 ml was transferred from the control tube at various times to other tubes, containing 0.5 ml 5 per cent sheep blood and 1.3 ml of PBS together with enough pyrosulphite to make the concentration of the reducing agent in these tubes equal to that in the other haemolytic systems. Haemolysis was similarly evaluated after 1 hr of incubation at 37°C after the addition of blood.

ASTA: Serum IN and IgG ASTA were diluted with PBS to approx. 0.2 ASTA U/ml. Then 0.5 ml of the dilutions were added to tubes containing 1.0 ml of PBS. The tubes were incubated at 37°C for temperature equilibration together with a series of tubes with 1.4 ml of PBS. After this 0.5 ml of staphylolysin (0.1 combining U/ml) in PBS

was added at various times to the tubes containing ASTA preparations and to a tube containing 1.5 ml of PBS at 37°C. Then 0.1 ml was drawn from this tube at various times and added to the tubes containing 1.4 ml of PBS. An 0.5 ml of 2 per cent rabbit red cells in PBS was immediately added to the 1.5 ml of toxin dilution and haemolysis was allowed to take place. Equal amounts of red cell suspension were added to the tubes containing ASTA preparations plus toxin, at various times after the addition of toxin to the tubes. In all systems, haemolysis was allowed to develop at 37°C for 1 hr and subsequently for 3 hrs. at +5°C.

Coprecipitation of streptolysin:

With the intention to study the presumed binding of streptolysin to the M-components AO and EN the following method was designed: Reduced and alkylated M-component preparations are incubated with excess streptolysin in the presence of carrier IgG of low AST activity. Subsequently carrier IgG, and reduced and alkylated IgG are immunoprecipitated by addition of an anti-IgG antiserum. If stable binding of streptolysin to the M-components occurs there is the possibility of coprecipitation of the lysin. To demonstrate the presence of lysin in the precipitates these are dissolved in a denaturing agent. When this is eliminated by dialysis against PBS, some recombination of the heavy and light chains from the M-component, previously dissociated by the denaturing agent, may occur. Since recombination is far from complete, as shown in Chapter IV, some lysin may remain free after dialysis and could then be assayed by its haemolytic activity.

The actual experimental procedure was as follows. Tubes with 0.5 ml of streptolysin received 100 µl of reduced, alkylated, radioiodinated IgG AO, EN, IN or GW. All additions contained the same amount of protein. IgG GW, not reduced, alkylated or iodine labelled, in 50 µl of PBS was then added as carrier. The ratio of carrier to reduced and

alkylated M-component in the mixtures was 20/1. Streptolysin was present in a calculated excess over the AST activities in the M-component preparations by a factor of at least 3. The mixtures were incubated for 30 min. at 37°C. Then 0.5 ml of heat treated (56°C, 30 min.) anti-IgG B serum was added. The tubes were kept at room temperature for 90 min. and subsequently overnight at +5°C. The resulting precipitates were washed twice with 1 ml of cold PBS. After careful removal of the last washing, 0.5 ml of 6 M guanidinium hydrochloride in PBS and containing BSA at 0.1 g/100 ml was added. The precipitates dissolved in a few minutes and the tubes were left at room temperature for $\frac{1}{2}$ hr. RIHSA in 10 µl PBS was added to each tube and the mixtures were transferred to dialysis bags; dialysis was carried out in the cold for 24 hrs. against a 1000-fold volume of buffer. In order to determine the efficiency of the immunoprecipitation, the sacks with contents were then assayed for radioactivity. The contents were subsequently centrifuged and the supernatants adjusted with PBS to a constant ^{131}I (RIHSA) radioactivity per ml. This adjustment was used to correct for differences in the loss of material in the bags. The preparations received the same volume of PBS and to the mixtures was then added 1/40 volume of 10 per cent sodium pyrosulphite. Reduction of streptolysin, if any, was carried out for 30 min. at room temperature. After reduction the preparations were serially diluted (dilution factor = 2) with PBS containing 0.2 per cent pyrosulphite. Haemolytic activity was assayed after the addition of 50 µl 5 per cent sheep red cells and incubation at 37°C for 60 min.

Inactivation of strepto- and staphylo-lysin by AST or ASTA.

Variation of AST: Closely spaced dilutions of serum AO, EN and SE, and of pooled normal IgG in PBS containing bovine serum albumin at a concentration of 0.1 g/100 ml (PBS—BSA) were prepared. Subsequently 0.5 ml of reduced streptolysin in PBS—BSA and con-

taining 1.0 combining U/ml was added to 1.0 ml of the AST preparations. The mixtures were incubated for 30 min. at 37°C, after which 0.5 ml of 5 per cent sheep red cells in PBS was added to the preparations. After a further 60 min. incubation, 2 ml of PBS were added to the tubes. After centrifugation haemolysis was evaluated by spectrophotometry of the supernatants.

In some experiments (with IgG preparations AO, EN and pooled normal IgG) the procedure was varied in that streptolysin was reduced with 1/40 volume of 10 per cent sodium pyrosulphite. PBS containing the same concentration of pyrosulphite but without BSA was used as diluent for reduced streptolysin and for dilution of the IgG preparations.

Variation of streptolysin: Serum AO, EN and SE and pooled normal IgG were diluted with PBS—BSA to 1 AST U/ml. The AST content of the preparation was calculated from the results of previous titrations on other aliquots of the same material. Streptolysin was reduced for 30 min. with 1/25 volume of 10 per cent sodium pyrosulphite. It was then diluted 5-fold. This preparation was further serially diluted with PBS—BSA containing the same sodium pyrosulphite concentration as the matter diluted, *i.e.* 0.08 per cent. To 0.5 ml aliquots of the AST preparations was then added 0.5 ml of PBS—BSA and the same volume of the streptolysin dilutions. Incubation of the mixtures and assay for haemolytic activity was carried out as in the experiments just described.

Variation of ASTA: 1.0 ml of closely spaced dilutions in PBS of IgG IN and IgG ASTA were prepared. The dilutions were mixed with 0.5 ml of staphylolysin (0.1 combining U/ml) in PBS and then incubated for 30 min. at 37°C. After the addition of 0.5 ml of 2 per cent rabbit red cells in PBS, the samples were further incubated for 60 min. at 37°C and subsequently for 3 hrs. in the cold. After centrifugation the supernatants were read at 525 nm for assay of haemolysis.

Variation of staphylolysin: 1.0 ml aliquots of IgG IN or IgG ASTA in PBS and of an ASTA

activity of 0.05 U/ml were mixed with 0.5 ml of different dilutions of staphylolysin in PBS. After incubation for 30 min. at 37°C 0.5 ml of 2 per cent rabbit red cells were added. Incubation for haemolysis and its evaluation was performed as in the preceding experiment.

Simultaneous variation of AST and streptolysin: 0.15 ml of dilutions of serum AO, EN and SE or pooled normal human IgG in PBS, each of an AST activity of 40 U/ml, were added to 1.0 ml of streptolysin, containing 8.3 combining lysin U/ml. The same volumes of the same materials, diluted 1/5 were mixed in centrifuge glass tubes, which also were used for the concentrated mixtures. Control mixtures of 1.0 ml of streptolysin with 0.15 ml of PBS and a control mixture of the same volumes of streptolysin dilution (1/5) in PBS, and PBS were prepared. The carefully stoppered tubes were incubated at 37°C for 30 min. and subsequently kept for 24 hrs. at +5°C. The concentrated mixtures were rapidly diluted 1/5 by addition of 4 volumes of PBS and all samples were assayed for haemolytic activity. For the assay 200, 100 and 50 µl from each sample was added to three tubes, containing a mixture of 1.0 ml of PBS and 0.5 ml of 5 per cent sheep red cells in PBS and sodium pyrosulphite at 0.25 per cent concentration. All tubes containing sheep blood were simultaneously incubated for 60 min. at 37°C then centrifuged and scanned for haemolysis by spectrophotometry at 525 nm.

Test for AST activity in mixtures of AST preparations and streptolysin: 5, 10 or 20 µl of serum SE, AO, EN or pooled normal human IgG all diluted with PBS to 40 AST U/ml were mixed with 40 µl of streptolysin, containing 9.8 combining lysin U/ml. The mixtures were incubated in stoppered tubes for 30 min. at 37°C. Subsequently 5 µl aliquots of the mixtures were examined for AST activity with the streptolysin blood-agar technique. Other aliquots (25 µl) were mixed with 50 µl of PBS containing 1/25 volume of 10 per cent sodium pyrosulphite. These mixtures were kept for 30 min. at room temperature. Then 25 µl of

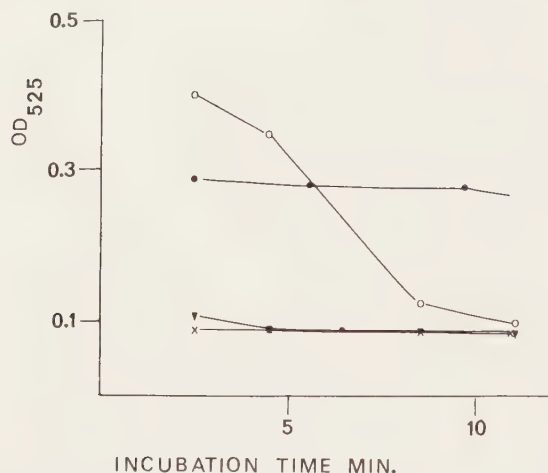


Fig. 1. Kinetics of inactivation of streptolysin by IgG AO, EN and pooled normal IgG. Mixtures of the AST preparations and a standard amount of reduced streptolysin were incubated for various lengths of time before addition of sheep red cells. The ordinate gives haemolysis as OD₅₂₅. Symbols: preparation AO -x-x-, EN -▼-▼- and normal IgG -o-o-. Also shown is haemolysis from streptolysin without addition of AST and amounting to 13.3 per cent of the lysin dose in the other tubes (●-●-).

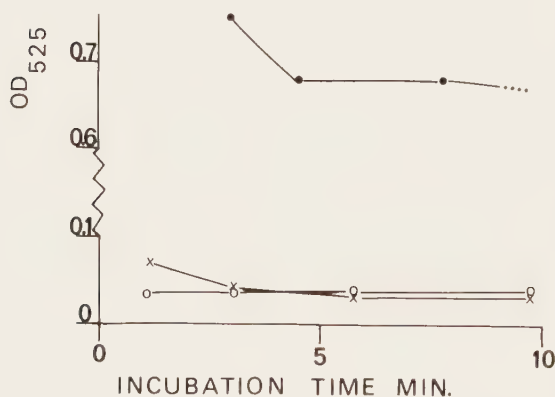


Fig. 2. Kinetics of inactivation of staphylolysin by IgG IN and pooled human ASTA active IgG (IgG ASTA). Mixtures of the IgG preparations and a standard amount of staphylolysin were incubated at 37°C for various lengths of time before addition of rabbit red cells. The ordinate gives haemolysis as OD₅₂₅, the abscissa the time of incubation before addition of blood. Symbols: IgG IN -x-x-, IgG ASTA -o-o-. The symbol ●-●- denotes haemolysis given by staphylolysin without addition of ASTA; the dose amounted to 5 per cent of that used with the ASTA preparations.

5 per cent sheep red cells in PBS was added and haemolysis was allowed to develop at

37°C for 60 min. Control mixtures of the AST preparations with PBS instead of streptolysin were similarly incubated and tested both for AST and haemolytic activity.

Test for ASTA activity in mixtures of ASTA preparations and staphylolysin. Serum In or IgG ASTA were diluted with 0.0175 M phosphate, pH 6.50 to a calculated ASTA content of 0.7 U/ml. Then 2, 5, 10 or 20 µl of the preparations were added to a dilution (0.5 combining U/ml) of staphylotoxin in PBS. Control preparations with PBS instead of staphylolysin were similarly prepared. All mixtures were incubated for 30 min. at 37°C. Subsequently, 5 µl aliquots were transferred to tubes, containing 25 µl of PBS plus 10 µl of 2 per cent rabbit red cells in the same buffer. Haemolysis was assayed after incubation at 37°C for 60 min. Other 5 µl aliquots of the mixtures were tested for ASTA activity with the rabbit blood-agar diffusion plate technique.

RESULTS.

Kinetics of streptolysin- and staphylolysin-inactivation by AST and ASTA.

AST: As seen (Fig. 1) the presence of IgG AO and EN resulted in a practically complete suppression of lytic activity already after incubation for 2 min. before the addition of blood. The effect of the pooled normal IgG seemed less pronounced. In all cases, within 6 min. of incubation haemolysis was lower than that from the lysin control. In another experiment with serum SE, AO, EN and the normal IgG there was a sharp drop of haemolytic activity in all samples with the exception of the one with the serum SE, which showed no definite haemolysis even in the sample with the incubation time of 2 min. In the other cases, samples incubated for 6 min., or for longer periods, gave less haemolysis than the lysin control. In experiments with serum EN, diluted to an AST activity of 2 U/ml, instead of 4 U/ml as in the experiments just described, no definite inhibition of haemolysis was noted.

ASTA: (Fig. 2). For both IgG ASTA and IgG IN, samples taken between 1 and 2

minutes after addition of lysin gave less haemolysis than the control lysin sample. A small continuous decrease in haemolysis was evident for the sample containing IgG IN. Decrease in haemolytic activity between 3 and 4.5 min. of incubation was observed in both of the duplicate samples of the lysin control preparations.

Coprecipitation of streptolysin.

When the dialysis sacks were counted, their content of ^{125}I radioactivity was found to amount to 68–77 per cent of that originally added with the reduced and alkylated IgG preparations. When the ^{125}I counts were normalised on the respective ^{131}I radioactivity figures, the values found differed by most 16 per cent of their means. When streptolysin was assayed the findings were clear cut. No haemolysis was noted in the tubes with IN and GW reduced and alkylated materials. The AO preparation gave haemolysis in the tubes 1, 2 and 3, the EN preparation in tubes 1 and 2. In a control experiment, where pyrosulphite was not included in the haemolytic system, no lysis was obtained.

Similar experiments were carried out using staphylolysin. In some of these, excess lysis was noted in preparations containing reduced and alkylated IgG IN. The difference from the control preparations with IgG AO, EN or GW, was, however, difficult to assess, since haemolytic activity was obvious in all preparations.

Inactivation of strepto- and staphylolysin by AST or ASTA.

Variation of AST. (Fig. 3a). The curve obtained with serum AO was less steep than that with the pooled normal IgG. A kinking of the curve in the region of 50 per cent haemolysis contrasted with the continuous sharp rise of the curve resulting from the normal IgG preparation. Serum EN gave a curve of quite a different form: a peak of haemolysis between lower values of it from samples of both higher and lower dilution. Further dilution (Fig. 3 b)

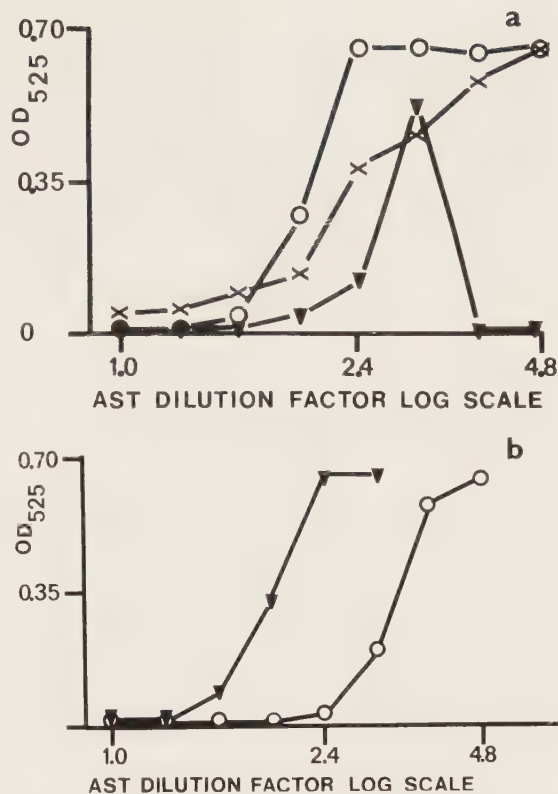


Fig. 3. Inactivation of streptolysin by varying doses of a) serum AO, EN, and pooled normal IgG, and b) serum EN and pooled normal IgG. Standard amounts of reduced streptolysin and serial dilutions of the AST preparations were incubated at 37°C and the mixtures subsequently assayed for haemolytic activity. The ordinate gives haemolysis as OD₅₂₅ and the abscissa the dilution factor for the AST preparations. In b) further dilutions of serum EN was employed. Symbols: AO -x-x-, EN -v-v-, IgG -o-o-.

resulted in a sigmoid curve almost congruent to that from normal IgG investigated in parallel. This prozonal phenomenon was also observed in other experiments with IgG EN and with IgG AO.

Depending on what part of the titration curve for serum EN that was used for determination of the AST dose giving 50 per cent haemolysis, 3 different titre values were obtained. These were: 130 000, 160 000 and 200 000 AST U/ml. In one ordinary titration, using doubling dilutions of IgG EN, the prozonal phenomenon was evident. Two specific AST activity figures were obtained,

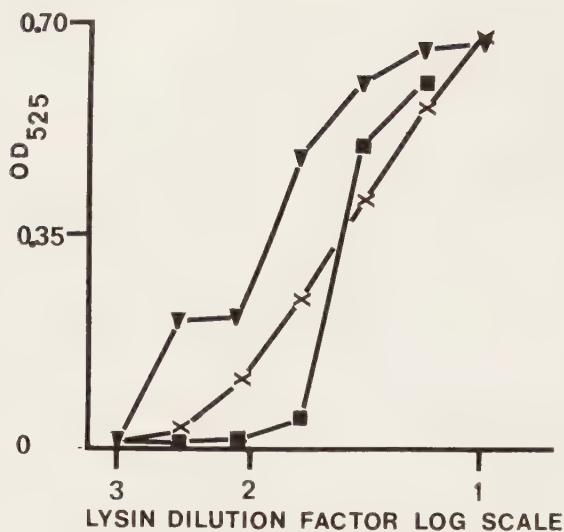


Fig. 4. Reaction between varying doses of streptolysin and constant amounts of serum AO, EN and SE. The AST preparations were mixed with varying doses of reduced streptolysin, incubated at 37°C and the mixtures assayed for haemolytic activity. Ordinate, haemolysis as OD₅₂₅; abscissa, dilution factor for streptolysin. Symbols: serum AO -x-x-, EN -v-v- and SE ■-■-.

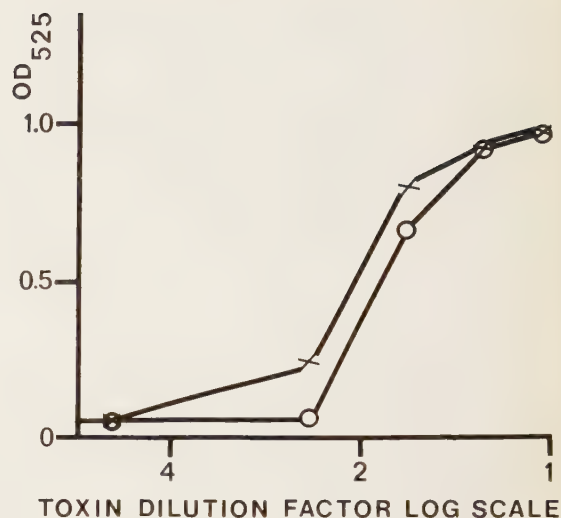


Fig. 6. Reaction between varying doses of staphylo-lysin and constant amounts of IgG IN and pooled ASTA active IgG (IgG ASTA). The IgG preparations were mixed with varying doses of staphylo-lysin, incubated at 37°C and the mixtures assayed for haemolytic activity. Ordinate gives haemolysis as OD₅₂₅ and abscissa the dilution factor for staphylo-lysin. Symbols: IgG IN -x-x-, IgG ASTA -o-o-.

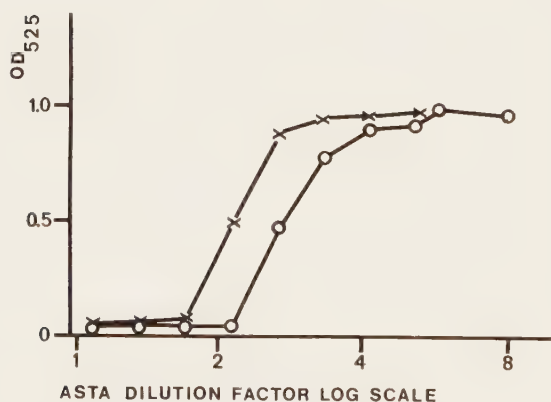


Fig. 7. Inactivation of staphylo-lysin by varying doses of IgG IN and pooled ASTA active IgG (IgG ASTA). Standard amounts of staphylo-lysin and serial dilutions of the IgG preparations were incubated at 37°C and the mixtures subsequently assayed for haemolytic activity. The ordinate gives haemolysis as OD₅₂₅ and the abscissa the dilution factor for the ASTA preparations. Symbols: IgG IN -x-x- and IgG ASTA -o-o-.

i.e. 1200 and 4300 AST U/OD₂₈₀/ml. At one occasion, duplicate determinations of IgG AO specific AST activity yielded the values 3200 and 3000, mean 3100 AST U/OD₂₈₀/ml. The

AST titre of serum AO as obtained from the titration curve was 240 000 AST U/ml.

Variation of streptolysin: (Fig. 4). Serum AO gave a curve, less steep than that from serum EN or SE investigated in the same experiment. In addition, serum EN showed an irregular pattern not noticed in the other titration curves. The normal pooled IgG yielded a curve similar to that of serum SE.

Variation of ASTA and variation of staphylo-lysin: (Fig. 5 and 6.) In both experiments employing IgG IN and IgG ASTA sigmoid curves were obtained. Both materials gave similar results, *i.e.* the shape of the curves did not differ noticeably.

AST activity in mixtures of serum AO or EN with streptolysin (Fig. 7): Inhibition of haemolysis was noted in repeated experiments with the mixtures containing streptolysin and an intermediate amount of serum AO or EN. When tested for haemolytic activity these mixtures gave a positive result. With lower concentration of AST material no definite inhibition of haemolysis was noted. Serum SE

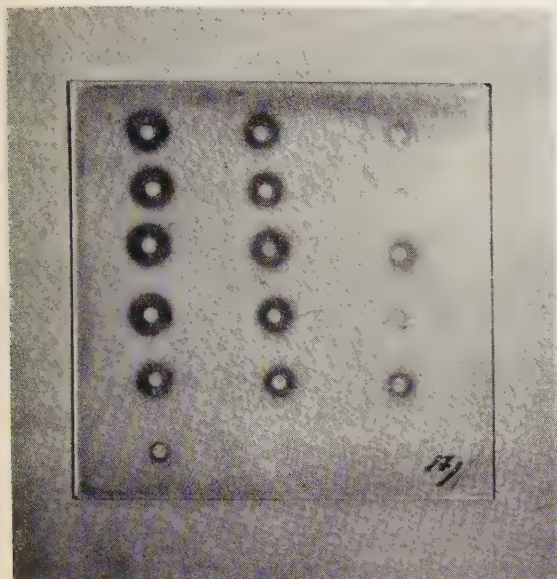


Fig. 7. Streptolysin blood-agar diffusion plate, test for AST activity in mixtures of normal IgG, serum AO or EN, and streptolysin. Row 1: from left to right, 20, 10 and 5 μ l of serum AO dilution plus PBS; Row 2: same serum volumes plus streptolysin. Row 3: same volumes of serum EN dilution plus PBS. Row 4: same serum volumes plus streptolysin. Row 5: same volumes of normal IgG solution plus PBS; Row 6: same volumes of IgG solution and streptolysin. Haemolytic activity was present in the mixtures of streptolysin with 5 μ l of IgG solution, 5 and 10 μ l of serum EN and with 5–20 μ l of serum AO. For further explanations, see the Text.

or the normal IgG preparation never resulted in inhibition of haemolysis in those cases where the mixtures showed haemolytic activity when tested after incubation. The control preparations without lysin gave no haemolysis.

ASTA activity in mixtures of serum IN or IgG ASTA and staphylolysin: Inhibition of haemolysis was not noted for mixtures giving haemolytic reactions.

Simultaneous variation of AST and streptolysin: The results obtained in the experiments with serum AO, EN, SE and with normal IgG are given in Table 1. There was a definite difference in haemolytic activity between the 2 systems, *i.e.* diluted and undiluted, when the monoclonal AST preparations were tested. The originally diluted system was shown to yield increased haemolysis. This difference was not noted with the polyclonal preparations. In

these, the diluted and undiluted preparations yielded the same degree of haemolysis or sometimes possibly a slightly higher haemolytic activity in the undiluted samples. The same findings were obtained with the streptolysin controls. As diluted and undiluted staphylolysin differed appreciably in haemolytic potency after incubation, similar experiments with ASTA material were not performed.

DISCUSSION.

The kinetic experiments with serum AO and EN showed that the shorter the incubation time of the mixtures before addition of the red cell suspension, the higher the degree of haemolysis. This finding implies, that constituents of the sera and streptolysin reacted, resulting in the blocking of the lytic enzyme activity before addition of the red cell suspension. From this one concludes that the M-components AO and EN block haemolytic activity from streptolysin by reaction with the toxin. For the mixtures of IgG IN and staphylolysin, the findings may suggest the

Table 1. *Haemolytic activity OD 525 in mixtures of AST preparations and streptolysin at 2 different concentrations of the reactants.*

	Preparation	Dilution step		
		1	2	3
I	Serum AO+lysin	0.453	0.112	0.010
	Serum AO+lysin, dilut.	0.595	0.552	0.192
	Serum EN+lysin	0.447	0.098	0.003
	Serum EN+lysin, dilut.	0.523	0.154	0.008
	Serum SE + lysin	0.591	0.380	0.034
	Serum SE + lysin, dilut.	0.545	0.325	0.027
II*	Serum AO+lysin	0.447	0.044	0.021
	Serum AO+lysin, dilut.	0.633	0.562	0.208
	Serum EN+lysin	0.340	0.071	0.017
	Serum EN+lysin, dilut.	0.448	0.270	0.023
	Serum SE +lysin	0.650	0.301	0.066
	Serum SE +lysin, dilut.	0.598	0.396	0.065
III	Normal IgG+lysin	1.116	0.988	0.449
	Normal IgG+lysin, dilut.	1.100	1.101	0.438
	Normal IgG+lysin	1.095	0.865	0.422
	Normal IgG+lysin, dilut.	1.104	0.915	0.376

Dilution step signifies the different amounts of the AST-streptolysin mixtures used in the assay of haemolytic activity. Roman numerals indicate sets of simultaneous determinations.

* denotes assay of haemolysis in duplicate.

same mechanism. There was a small, but definite, drop in haemolytic activity in the mixtures taken during the first few minutes of incubation. However, as heating of staphylolysin at 37°C seemed to result in some inactivation of the toxin, as shown by the toxin control, this conclusion is not unequivocally valid for the IN material. If a reaction between the M-component and the staphylolysin was operative and if this reaction was rapid, as suggested by the experimental findings, the slow lysis from staphylolysin should make the time course of the inactivation reaction difficult to establish. In the case of serum AO, EN, and SE and the pooled normal IgG the experimental results showed that at least 86 per cent of the streptolysin was inactivated within 6 min. The results with IgG IN and IgG ASTA suggested that more than 98 per cent of the staphylolysin had been blocked during the first 1.5 min. of incubation of the ASTA-toxin mixtures.

The coprecipitation experiments demonstrated that more streptolysin activity was recovered from the precipitates containing reduced and alkylated IgG AO or EN than from those made up with reduced and alkylated IgG IN or GW; *i.e.* the effect was evidently correlated with the presence of AST active material. Since, as concluded above, IgG AO and EN do react with streptolysin, the result of the coprecipitation experiment is almost certainly due to that reaction. Barring the unlikely possibility that streptolysin is modified in the reaction with the M-components so as to make the lysin unspecifically stick to immunoprecipitates, one can conclude that permanent binding occurs between the AST-active M-components and streptolysin. The findings in the experiments with IgG IN and staphylolysin were too unspecific to allow any definite conclusions.

In the experiments with variation of streptolysin or AST activity in the titration systems, IgG AO and EN gave results departing from those obtained with the polyclonal AST preparations. In the variation of AST content, the EN material gave rise to a prozonal pheno-

menon: the lytic activity could be demonstrated in a narrow concentration interval when the antibody was present in excess. The same finding was obtained with IgG AO. It may be that haemolytic complexes between the M-components and streptolysin are formed at antibody excess. The prozonal phenomenon introduces ambiguity in the AST titrations of the AO and EN materials. Three different titre levels may be obtained during titration; *i.e.* depending on whether the highest haemolysis used for interpolation of the 50 per cent haemolysis end point corresponds to the ascending or the descending part of the prozonal haemolysis peak in the titration curve, or to the sigmoid part of the curve, obtained at higher dilutions of the M-component. Erroneously low titre values may evidently be obtained using the ordinary titration method. This may explain the low recovery figures which were found (Chapter II) when the measured specific AST activities of the M-components AO and EN were compared with the calculated values obtained from serum titres and contents of M-component. Using the highest specific activity found for IgG EN, *i.e.* 4300 AST U/OD₂₈₀/ml, and the concentration of M-component in serum EN A, *i.e.* 3.8 g/100 ml (Chapter II), leads to an estimate of serum titre of 220 000 AST U/ml. This is almost exactly the value obtained from the titration curve for serum EN, *i.e.* 200 000 AST U/ml. Similar calculations with the highest value of IgG AO specific activity (3100 AST U/OD₂₈₀/ml) yields a serum titre of 210 000 AST U/ml which may be compared with the result from the titration curve, *i.e.* 240 000. Since a prozonal phenomenon was not demonstrated in the case of IgG or serum IN the discrepancy between the specific ASTA activities directly measured, and those calculated from the serum titre and M-component concentration, respectively, remain unexplained.

When the amount of streptolysin was varied in the titration system, serum AO gave a less steep response in haemolysis than noted with serum EN, SE or the pooled normal IgG.

Compared to polyclonal AST globulin molecules, this may not necessarily indicate abnormality, since there may exist a sub-population of polyclonal AST active molecules which give a dose response curve of the type encountered with AO material; such molecules, together with a majority of other AST active globulins yielding a "normal polyclonal" response curve, may not be able to contribute significantly to the result of the titrations. The irregularity in the titration curve noted with serum EN, if outside the experimental errors, may be interpreted along the same line. With IgG IN there was no obvious deviation from the results encountered with the pooled normal ASTA active IgG preparation.

AST activity could be demonstrated in some mixtures of streptolysin and serum AO or EN, in spite of the demonstrated lytic activity in the preparations. This type of finding was not obtained with serum SE or pooled normal IgG; this phenomenon was not demonstrable for mixtures of staphylolysin and ASTA active preparations, whether mono- or polyclonal. The result with serum AO and EN could be given several alternative explanations:

1. Free lysin and free M-component may exist together in the mixture. It is probable that the AST-streptolysin reaction had proceeded almost to equilibrium because the incubation time chosen (30 min.) was far in excess over the time for combination, suggested by the kinetic experiments. If this was the case the findings imply that the affinity between streptolysin and the M-components is appreciably lower than that between lysin and the polyclonal AST globulins investigated;

2. The lytic activity of the mixtures may be due to the appearance of haemolytic complexes between the M-components and streptolysin. In this case the complexes are either unstable enough to dissociate during diffusion in the blood agar-streptolysin plate resulting in liberation of free M-component; or the complexes are stable and capable of binding more streptolysin, resulting in a decrease in haemolysis in the diffusion plate. From the first interpretation one could again infer that the

M-component-streptolysin binding is comparatively weak. The second alternative is compatible with stable binding.

When AST and streptolysin were simultaneously varied the inhibition of lytic activity was found to be markedly concentration dependent in the preparations with serum AO and EN. This effect was not obtained with the polyclonal preparations. Since the haemolytic activity of the streptolysin control preparations was practically unaffected by changes in concentration the findings suggest that the reaction between the M-components and streptolysin was perceptibly influenced by variations in the dilution of the system. If this really was the case, the binding between M-component and lysin must be comparatively weak and probably weaker than that between polyclonal AST globulin and streptolysin. In the well-studied BSA-anti-BSA system presence of a dilution effect was shown to correlate with a low antigen-antibody affinity (Farr 1958). Characteristically the early primary antibodies were of low affinity compared to the secondary response antibodies and the same has been found for anti-hapten IgG antibodies (Eisen and Siskind 1964). Such findings may exemplify a general principle of differences in the quality of early and late antibodies (last ref.). Thus it may be that the AST active M-components AO and EN are more similar to early antibodies than the polyclonal AST preparations used for comparison.

SUMMARY

With the techniques used rapid binding between the AST active M-components AO and EN and streptolysin was demonstrated. The findings suggested that the M-components were more weakly bound to streptolysin than the polyclonal AST preparations used for comparison. A prozonal phenomenon was observed in AST titrations of the M-components. It may explain differences found between specific AST activities of purified M-components and calculated specific activities of M-components in serum.

Concluding remarks

The findings of the foregoing Chapters show that the M-components AO, EN and IN contain serological activity of the specificity found for the whole sera. For the M-components AO and EN the apparent AST activity was shown to result from binding between the M-components and streptolysin. Binding of staphylolysin by the M-component IN was not unequivocally proved but is considered probable in view of the collected experimental results. These M-components fulfil the structural requirements of antibody activity investigated for, *i.e.* localisation of the serological activity to the Fab (Fd) part of the molecule and formation of active composite heavy and light chain molecules at recombination of their chains.

Antibody activity in the Fab fragments and reconstitution of antibody active molecules from isolated polypeptide chains are, however, not sufficient criteria to establish the M-components as normal antibody globulins. Even a pathologic structure, reacting with strepto- or staphylolysin may be localised on the Fab fragment; and there is no obvious objection to reformation of an abnormal combining site structure at recombination of the polypeptide chains.

Since the diagnosis of the patient AO was myeloma, *i.e.* a disorder of excessive proliferation of plasma cells, this indicates that his M-component had been produced in such cells. This type of argument fails in the other cases, where this diagnosis has not been established (Waldenström 1967, Hällén et al. 1968). The fact, however, that the M-components consisted of both heavy and light polypeptide chains in all cases implies immunocytes as the producers of these globulins, and this is postulated for the following discussion.

If it is assumed that the M-components AO and EN have the same specificity as the bulk of normal AST globulins the experimental findings suggest that the binding affinity of the M-components is lower than that of normal AST. This does not exclude these M-components as normal antibodies. As often demonstrated (Jerne 1951, Jerne and Avegno 1956, Farr 1958, Eisen Siskind 1964, Svehag 1965) normal antibodies display a wide variety of combining affinities. According to selective antibody theories this should imply that some clones of immunocytes produce low affinity antibodies. Thus there is the possibility, that the M-components AO and EN represent normal products of low affinity clones. Low affinity may also be well compatible with a pathologic structure of the antibody combining site. It may be surmised, that a more or less random production of configurations in a pathologic process should more often than not imply a bad fit with the antigenic configuration. The finding of a G-myeloma protein with anti-hapten activity (Eisen et al 1967) is of considerable interest, since in this case the affinity for the antigen was directly measured. The figure given for the intrinsic association constant was considerably lower than values for the average intrinsic association constant for early, low affinity rabbit antibodies against the same antigenic configuration (ϵ -2, 4-dinitrophenyl-L-lysine) published from the same laboratory (Eisen and Siskind 1964).

In conclusion, the M-components under study have properties characteristic of ordinary antibodies. This strengthens the belief, that they represent ordinary antibody molecules. The deviations in behaviour from that of normal polyclonal antibodies seen in some experi-

ments may reflect heterogeneity in the normal material.

If low affinity against the antigen is a common feature of antibody active IgG M-components, the detection of their activity may be very dependent on the sensitivity of the assay system. Anti-enzyme specificity of M-

components should be advantageous in this respect, since enzymes are possible to assay in low concentration. This may explain the remarkable predominance of AST activity among the known antibody-active IgG M-components, especially since AST titration is common in clinical practice.

Summary

The purpose of the present investigation of 3 human sera of which 2 (AO and EN) contained an excessive antistreptolysin-O (AST) activity, the 3rd (IN) an extremely elevated anti- α -staphylolysin (ASTA) activity, and all IgG M-components, was the following: to establish whether the M-components had serological activity, if so, how the activity was localised in the globulin molecule. Further if their polypeptide chains could be recombined to serologically active composite molecules. Finally whether the M-components could bind their presumed antigens.

Chapter I. By modification of previously known techniques there was developed an acrylamide gel electrophoresis system, suitable

for characterisation of polypeptide chain preparations. A low molecular marker was found useful for standardisation of results obtained at gel filtration. Microtechniques based on gel diffusion and suitable for assay of antistreptolysin or antistaphylolysin activity were designed. These techniques were used in the following investigations.

Chapter II. Antibody activity was demonstrated in the M-components by the following criteria: electrophoretic distribution of the serum IN ASTA activity; inhibition of the antibody activity of the sera with antisera against IgG and against the antigenic light chain type of the respective M-components but not against the respective alternative types;

correlation of the specific antibody activity of ion exchange chromatography fractions of the sera with the fractions' content of M-component.

Background IgG was found to be of negligible specific antibody activity compared to that of the M-components. The 2 AST active M-components were of alternative light chain antigenic types, the ASTA active M-component of type L. The M-components were of different Gm types. These were in all cases compatible with the heavy chain subgroup IgG 1.

Serum AO and EN AST activity was sensitive to heating at 56°C and the serum AO activity also sensitive to dialysis against low ionic strength phosphate buffer.

Chapter III. Proteolytic fragments were obtained from the 3 M-components by gel filtration and preparative electrophoresis of papain digests. Antibody activity was not demonstrated in the Fc fragments but was present in the Fab fragments. For the case AO an unidentified component, possibly an Fd fragment may also contain antibody activity.

Chapter IV. Heavy and light chains were prepared from the radio-iodine labelled M-components. In all cases antibody activity was demonstrated in heavy chain preparations. From the results at acrylamide gel electrophoresis of the chain preparations, contamination of the heavy chain preparations with M-component or light chains could not be ruled out as the cause of the antibody activity in the heavy chain preparations. An apparent antibody activity in the light chains AO was caused by contaminating material, provision-

ally identified as Fab fragments. In autologous recombination experiments, potentiation of antibody activity was demonstrated in all cases as well as formation of antibody active composite heavy and light chain molecules. Antibody active hybrid molecules were formed in the 2 homologous crosses between chains from the M-components AO and EN. Antibody activity could not be demonstrated in hybrid molecules created in other cross experiments among them with chains from the M-components AO and IN.

Chapter V. Binding between streptolysin and the M-components AO and EN was demonstrated. Suggestive evidence for binding of staphylolysin by the M-component IN was also obtained. The affinity of the AST active M-components for streptolysin was judged to be comparatively low as suggested by the finding of a concentration dependence of the streptolysin-AST reaction which was not obvious in the same type of reaction between lysin and polyclonal AST.

A prozonal phenomenon was obvious in AST titration of serum or M-component AO and EN. The phenomenon may explain discrepancies often observed between specific AST activities of purified M-components and specific activities calculated for the M-components in serum.

In conclusion, the M-components under study have properties characteristic of ordinary antibodies. This strengthens the belief, that they represent ordinary antibody molecules. The deviations in behaviour from that of normal polyclonal antibodies seen in some experiments may reflect heterogeneity in the normal material.

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